THE UNIVERSITY OF ALBERTA

NITROGEN METABOLISM IN SHEEP

BY

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ABSTRACT

Experiments were conducted to study the metabolism of nitrogenous compounds in the rumen and to examine the interrelationships between rumen and animal tissue metabolism.

In one series of experiments, $^{15}NH_4Cl$ was continuously infused into the rumen of sheep until steady state concentrations of isotope were achieved. It was determined that an amount of nitrogen equivalent to 60 to 92% of the daily intake was transformed into ammonia nitrogen in the rumen. Results obtained by comparison of steady state concentrations of ^{15}N in the microbes with that of rumen ammonia indicated that from 50 to 65% of the bacterial nitrogen and from 31 to 55% of the protozoal nitrogen were derived from rumen ammonia *in vivo*.

Microbial growth in the rumen resulted in the assimilation of from 1.7 to 2.6 g of nitrogen for every 100 g of dry matter fermented in the rumen *in vivo*.

The possibility of fixation of nitrogen gas in the rumen was examined by incubating rumen contents and washed microbial cells from the rumen with $^{15}N_2$ under anaerobic conditions. No fixation of gaseous nitrogen was detected.

In one experiment a mixture of ^{15}N-ammonia and ^{14}C-urea was infused into the portal vein of sheep to study the metabolism of ammonia by the liver. It was found that approximately 93% of the ammonia which was absorbed from the digestive tract of the sheep was metabolized to urea in the liver when the sheep was fed a hay diet of high protein content.

The arteriovenous differences of various metabolites across the rumen were measured and these differences were related to that of

acetate in order to obtain estimates of the quantities of the metabolites absorbed into ruminal blood. It was found that from 4.8 to 9.9 g ammonia nitrogen entered the rumen blood daily. Glycine and glutamate nitrogen were absorbed into rumen blood in significant amounts in two of five experiments.

The oxygen consumption (STP) of the rumen tissue of three sheep was estimated to be from 12 to 59 ml O_2/min or from 14 to 70 ml O_2/min/kg rumen tissue.

The need for further studies into the factors affecting the efficiency of microbial growth in the rumen, ruminal blood flow, amino acid absorption and ruminal oxygen consumption was pointed out.

ACKNOWLEDGEMENTS

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SECTION I

THE METABOLISM OF ^{15}N-AMMONIA AND THE SYNTHESIS OF MICROBIAL NITROGEN IN SHEEP*

INTRODUCTION

The ruminant animal is unique in that it can survive and produce when fed a diet free of proteins and amino acids (Loosli, Williams and Thomas, 1949). The ability of the animal to exist without protein is a result of the action of the microorganisms in the rumen which utilize non-protein nitrogen for the synthesis of essential and non-essential amino acids (Hungate, 1966).

Little information was available in the literature concerning the quantitative aspects of nitrogen metabolism in the rumen when this investigation was undertaken. Hungate (1966) stated that the degree of utilization of rumen ammonia for the synthesis of nitrogenous materials was 'one of the most intriguing problems in rumen ecology'. Similarly, Milligan (1967) pointed out that available information in the literature suggested that the growth of the ruminant animal was limited by the extent of microbial protein synthesis in the rumen.

The experiments described in Section I were thus designed to gain information pertaining to the quantitative aspects of rumen microbial ammonia utilization and growth, as well as other aspects of nitrogen metabolism in sheep. It was hoped that the information obtained in this study would contribute to the understanding of nitrogen metabolism in the ruminant animal, and that this knowledge would be useful in elucidating the

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* Section I of this thesis was published in a similar form: Mathison, G.W. and L.P. Milligan. 1971. Nitrogen metabolism in sheep. Brit. J. Nutr. 25: 351-366.

nutritional requirements of these animals and in evaluating and modifying existing feeding practices.

LITERATURE REVIEW

A. The production of rumen ammonia

Ruminant animals normally ingest most of the nitrogen they require in the form of plant protein, and this protein is a major source of rumen ammonia (Hungate, 1966). Protein which enters the rumen is initially digested by microorganisms to peptides and amino acids (Abou Akkada and Blackburn, 1963). These compounds are incorporated into microbial material, or further metabolized to ammonia, carbon dioxide and organic acids (Hungate, 1966). Protein is generally hydrolyzed to amino acids at a slower rate than the resultant amino acids are incorporated into microbial material or converted to ammonia; as a result there usually is a relatively high concentration of ammonia and a relatively low concentration of amino acids and peptides in the rumen (Annison, Lewis and Lindsay, 1959; Lewis, 1962).

Most of the proteolytic activity in the rumen has been found to be in or associated with, the microbial cells rather than the rumen fluid (Hungate, 1966). Blackburn and Hobson (1960a) isolated bacteria from the rumen and found that proteolytic activity was present in rumen organisms at all times, even though the activity did increase after feeding. Annison (1956) suggested that the proteolytic capacity of bacteria was not greatly affected by the diet. From these observations it would appear that most proteins would be subject to extensive degradation in the rumen.

Measurements have, in fact, shown that a large proportion of the dietary protein is attacked by rumen microorganisms. Weller, Pilgrim and Gray (1962) found that 80% of the ingested plant nitrogen was converted to microbial nitrogen in sheep, and that approximately 80% of the plant protein was degraded in the rumen. McDonald and Hall (1957) found that 97% of

ruminally administered casein was degraded before leaving the rumen. Zein, however, was only degraded to the extent of approximately 40% in the rumen before passing into the lower digestive tract (McDonald, 1954). Annison (1956) concluded that the extent of protein digestion in the rumen increases as the solubility of the protein increases.

Ammonia in the rumen is derived from urea in addition to plant protein (Huhtanen and Gall, 1955). Urea normally enters the rumen in the saliva, and the concentration of urea in the saliva reflects the nitrogen level of the diet (Somers, 1961a). The amount of nitrogen which enters the rumen ammonia pool by this route has been estimated at 700 mg daily (Somers, 1961b). Urea also enters the rumen by diffusion across the rumen wall, and Houpt (1959) found that 3.2 g of nitrogen entered the rumen of a sheep daily in this manner. This result agrees quite well with results obtained by Weston and Hogan, (1967), Cocimano and Leng (1967) and Ford and Milligan (1970), who found that from 3 to 9 g of urea nitrogen per day were recycled to the digestive tract of sheep; presumably a large portion of this was to the rumen.

Various non-protein nitrogen sources besides urea yield ammonia when they are metabolized in the rumen (Blackburn, 1965). It would thus appear that a large portion of the nitrogen-containing material that a ruminant animal eats is digested in the rumen and that a major end product of this metabolism is ammonia. This indicates that ammonia metabolism does play a significant role in the nitrogen economy of the ruminant animal.

B. Utilization of ammonia by rumen microorganisms

Direct evidence for the utilization of ammonia by rumen microorganisms has been obtained by observing the incorporation of ^{15}N-ammonia into

microbial cells (Land and Virtanen, 1959; Ulbrich and Scholz, 1963; Ulbrich and Scholz, 1966a).

Allison (1969) suggested that ammonia is probably the most important source of nitrogen for rumen bacteria. This conclusion was based upon results such as those obtained by Bryant and Robinson (1962), who found that 82% of isolated rumen bacterial strains utilized ammonia as the main source of nitrogen. Similarly, Allison, Bryant and Doetsch (1959) suggested that certain rumen bacteria utilized ammonia in preference to exogenous organic nitrogen. This greater utilization of ammonia as compared to amino acids by microbes is probably due to the absence of, or the low activity of systems for the transport of amino acids in rumen bacteria (Allison, 1969).

Various enzymatic systems are present in rumen microorganisms for the fixation of ammonia nitrogen. It is probable, however, that the glutamic dehydrognase system is the main ammonia fixing mechanism in rumen bacteria (Allison, 1969). Palmquist and Baldwin (1966) found both NAD-linked and NADP-linked glutamic dehydrogenases (EC 1.4.1.2 and 1.4.1.4, respectively) in mixed rumen microorganisms, and also observed that the latter enzyme system tended to increase when increasing amounts of concentrate were fed.

Chalupa et al. (1970a) demonstrated the presence of other enzyme systems capable of fixing ammonia. Glutamine synthetase (EC 6.3.1.2) and carbamate kinase (EC 2.7.2.2) were found in rumen contents, although the workers found the latter enzyme was present in such low quantities that it was suggested that it could only be of minor importance in ammonia assimilation.

C. The production of microbial protein in the rumen

A large portion of the dietary nitrogenous material is digested in the rumen (Gray, Pilgrim and Weller, 1958) and a large percentage of the digested nitrogen is incorporated into microbial protein (Weller, Pilgrim and Gray, 1962), thus the nitrogen requirements of the ruminant animal are presumed to be met largely by microbial protein. The amount of microbial growth which occurs in the rumen is, thus, of considerable importance in the nitrogen metabolism of the ruminant animal.

Microbial growth in the rumen is limited thermodynamically by the anaerobic conditions which exist in the rumen; as a consequence only a limited quantity of nitrogenous material can be synthesized into cellular material (Hungate, 1966). Since the host animal is aerobic, the quantity of microbial material synthesized in the rumen may be insufficient to meet the protein requirements of the animal (Milligan, 1967).

The amount of microbial growth which occurs under anaerobic conditions has been examined. Bauchop and Elsden (1960) suggested that 1 mole of ATP produced by fermentation would yield 10 g of cellular material under anaerobic conditions and that this value was relatively constant. Hungate (1963), however, found microbial cell yields varied with the culture conditions and that microbial nitrogen incorporation was 1.2 to 2.2% of the substrate fermented in batch and continuous cultures respectively. Hobson and Summers (1967) found yields for the rumen microorganism Bacteroides amylophilus in continuous culture systems were similar to the higher values reported by Hungate (1963) and that the growth rate of the microorganisms influenced the quantity of cells produced.

Few direct measurements of microbial growth in the rumen had been

made at the time this work was originally submitted for publication. Conrad and Hibbs (1968) used the amount of methionine synthesized in the rumen as an indicator of microbial growth to estimate that 1.63 g of nitrogen was incorporated into microbial material for every 100 g of dry matter fermented in the rumen of a cow. Hungate (1966) used published results of various nitrogen metabolism studies in ruminants to estimate that the average microbial growth in the rumen was equivalent to 1.1 g of nitrogen assimilated for each 100 g of substrate fermented.

Several reports on the yield of microbial cells in the rumen have been published since this work was submitted for publication. Purser (1970) reviewed work which indicated that the incorporation of nitrogen into rumen microorganisms could be considerably greater than the average of 1.1% of the substrate fermented as suggested by Hungate (1966). Walker and Nader (1970) estimated that 2.3 g of microbial nitrogen was formed per 100 g of organic matter digested in the rumen from *in vitro* results using radioactive sulfide incorporation to measure growth. A very high cell yield of 3.7 g of microbial nitrogen incorporated for every 100 g of organic matter digested in the rumen was obtained by Hume (1970) in sheep fed semi-purified diets.

In summarizing the available literature on microbial growth in the rumen it can be concluded that the early estimate of approximately 1.1 g of nitrogen assimilated for every 100 g of dry matter digested now appears to be less than the potential for growth in the rumen. Recent estimates and measurements indicate that microbial growth in the rumen can be more efficient than this figure would suggest.

EXPERIMENTAL

A. Methods

$^{15}NH_4Cl$ was continuously infused into the rumen of the sheep in order to achieve a relatively constant enrichment of the rumen ammonia pool under steady state conditions. Bacteria, protozoa, ammonia and other metabolites were collected, isolated and analyzed for their ^{15}N content to allow the assessments of nitrogen interconversions to be made by tracer methodology. The quantity of material passing the abomasum daily was also estimated by the use of lignin and polyethylene glycol (PEG) as markers, and the quantity of microbial material synthesized in the rumen was estimated using this value and the ^{15}N concentration of the abomasal material.

B. Animals and feeding regimen

A Southdown ewe and a Suffolk wether weighing approximately 45 and 55 kg, respectively, and both about 2 years of age, were fitted with ruminal and abomasal fistulas at least 2 months before the first experiment was conducted (see Appendix A for a description of the technique used in fitting the animals with abomasal fistulas). For at least 2 weeks before and during each experiment, the animals were provided with food for 2 out of every 10 minutes from a timed, moving belt feeder (Ford, 1969). Water was given by continuous infusion into the rumen. The experimental chamber was maintained at 18 to 20 C, with continuous lighting to help maintain steady environmental conditions. Diets 1 and 2 were chopped native grass hays which contained 1.4 and 1.6% nitrogen respectively (dry matter basis). Other diets were rolled barley and a chopped brome-alfalfa hay (high-protein hay) which contained 1.8 and

2.5% nitrogen respectively on a dry matter basis.

C. Infusion and sampling procedures

Approximately 40 mg of ^{15}N daily, in the form of $^{15}NH_4Cl$ (99 atoms % ^{15}N; Bio-Rad Laboratories, Richmond, California) were continuously infused into the middle of the rumen contents of a sheep together with approximately 8 g of PEG in a total volume of 2 litres of water. Samples (100 - 150 ml) of digesta were taken from the rumen and abomasum at 12 hour intervals, and bacteria, protozoa and rumen ammonia were isolated for ^{15}N determinations. Samples were obtained from several sites in the rumen by the use of a vacuum pump and a rubber tube, and were mixed to yield composite preparations.

D. Separation of bacteria and protozoa

The differential centrifugation method of Blackburn and Hobson (1960b) was used to separate bacteria and protozoa (see Appendix B). Abomasal contents were used for the separation of bacterial and protozoal fractions derived from the low-protein diets 1 and 2 and for the preparation of the bacterial fraction from the sheep given the high-protein hay diet. In the trials entailing measurement of passage of material through the abomasum, rumen contents were used for the separation of the protozoa derived from the high-protein hay and of the protozoa and bacteria derived from the barley diet. This was done to reduce, as far as possible, interference with the normal flow of digesta through the abomasum. Three comparisons of the content of ^{15}N in comparable microbial preparations from the abomasum and the rumen showed that the site of sampling did not influence the value obtained.

E. Estimation of plant material passing the abomasum

The residues from at least four abomasal samples, obtained by straining the abomasal contents through cheese-cloth and washing with water on the cloth to remove all protozoa and loosely bound bacteria, were analyzed for nitrogen, lignin and ^{15}N for the trials in which abomasal flow as measured. It was assumed that all of the excess ^{15}N in the residue was in bacterial cell material. The proportion of the nitrogen in the residue that was bacterial was then estimated as

$$\frac{\text{Excess } ^{15}N \text{ per unit of N in residue}}{\text{Excess } ^{15}N \text{ per unit of N in isolated bacteria}} \times 100\%$$

where the isolated bacterial values refer to those determinations on bacterial samples isolated in the same trial. The remaining portion of the nitrogen in the washed residue was considered to be of plant origin. The amount of the dry matter in the sample which was of plant origin was considered to be the difference between the total dry matter and the microbial dry matter, the latter being calculated from the amount of microbial nitrogen in the sample and the amount of microbial dry matter per unit of microbial nitrogen. It was then possible, with this information and a measurement of the lignin in the residue, to calculate the ratios of plant nitrogen to lignin and plant dry matter to lignin in the undigested food material in the abomasum and thus to estimate the flow of plant material passing through the abomasum by treating lignin as an indigestible marker (Hogan and Weston, 1967a).

F. Estimation of soluble nitrogen passing the abomasum

PEG was used as a reference marker to estimate the amount of soluble nitrogen passing the abomasum as well as to check the values based upon lignin determinations. The supernatant liquids from the abomasal contents separated by the above procedure were used for the determination of PEG and soluble nitrogen.

G. Analytical methods

Ammonia in rumen liquid was prepared for isotopic analysis by distillation from centrifuged rumen fluid made basic by the addition of an excess of 2N KOH. The total concentration of base in the distillate, which would include methylamine as well as ammonia, if methylamine was present in the rumen liquid, was determined by titration and was compared with the concentration of ammonia determined by the method of Fawcett and Scott (1960), which does not measure aliphatic amines (see Appendix F for an outline of the method of Fawcett and Scott). This comparison showed that over 99% of the volatile base in rumen liquor was ammonia.

Microbial protein was isolated for ^{15}N determinations by the method of Roberts et al. (1955) (Appendix C).

^{15}N was measured on a mass spectrometer (CEC 21-614 RGA) using gaseous nitrogen prepared for analysis under vacuum by the addition of sodium hypobromite to ammonium sulfate according to the procedure of Mulligan and Workmall (1959) (Appendix D). Samples of rumen ammonia and microbial protein were taken from each trial to ascertain if there was any accumulation of ^{15}N between trials; there was no evidence of any such accumulation. The mean of the pre-trial abundance by our method of determination was 0.3475 atoms percent ^{15}N with a standard deviation of $\pm$0.0023. This

value was not corrected.

Total nitrogen was determined by the Kjeldahl method, and combustible energy by the oxygen bomb calorimeter. Samples were dried to constant weight at 105 C under reduced pressure for dry matter determinations. PEG was estimated by the method of Smith (1959b), with turbidity readings at 525 nm after 20 minutes (this procedure is outlined in Appendix F).

Lignin was determined by the 72% (w/w) H_2SO_4 method of Norman and Jenkins (1934), as described in Appendix F. In agreement with the suggestions of Badawy et al. (1958) and Hogan and Phillipson (1960), no digestion of lignin took place in the rumen of the sheep as determined by comparing calculated flow-rates based upon lignin passage through the abomasum and the flow-rates of abomasal material estimated with PEG.

RESULTS

Table 1 summarizes the feed intake and nitrogen balance data of the sheep used in the experiments. The small differences between the intake of digestible energy by the sheep make it possible to compare the effect of different dietary nitrogen contents and types of feed on the nitrogen metabolism of the sheep.

A. Microbial utilization of rumen ammonia

The enrichment of the bacterial, protozoal, urinary and fecal samples with ^{15}N is shown in Figure 1 for each of the four diets. The transfer quotients, obtained by expressing the concentrations above natural abundance of ^{15}N in these fractions at steady state as a percentage of the rumen ammonia enrichment, indicate the proportion of the nitrogen in the fraction that was derived from rumen ammonia (Table 2). The transfer quotients show that from 50 to 65% of the bacterial protein nitrogen was derived from rumen ammonia and that the concentration of isotope in the total nitrogen of the bacteria was only slightly higher than the ^{15}N enrichment of the protein nitrogen fraction. Since the experimental methods were such that only ammonia which equilibrated with extracellular rumen ammonia would be measured as contributing to bacterial nitrogen, these transfer quotients represent a minimum range for the utilization of ammonia by bacteria. As the concentrations of rumen ammonia increased when the hay rations were given to the sheep, there may have been a decrease in the proportion of ammonia utilized by bacteria (Figure 2), however, measurements were made at only three concentrations of rumen ammonia.

Nitrogen derived from rumen ammonia formed 31 to 55% of the protozoal

Table 1. Intake, output and apparent digestibility of dry matter, energy and nitrogen, and nitrogen retention of sheep.
(Mean values with their standard errors* for total daily collection from each sheep)

Diet	Dry matter			Gross energy				Nitrogen				
	Feed	Feces	Digestibility	Feed	Feces	Digestibility	Urine	Feed	Feces	Digestibility	Urine	Retained
	(g/day)	(g/day)	(%)	(kcal/day)	(kcal/day)	(%)	(kcal/day)	(g/day)	(g/day)	(%)	(g/day)	(g/day)
Low-protein hay 1	847	333±13	60.7±1.5	3618	1483±55	59.0±1.5	88±8	12.1	5.0±0.2	58.7±1.6	5.0±0.3	2.1±0.4
Low-protein hay 2	839	308±8	63.3±1.0	3610	1344±32	62.8±0.9	98±18	13.6	4.6±0.1	66.1±0.7	6.6±0.7	2.4±0.7
High-protein hay	667	222±4	66.7±0.6	2868	872±18	69.6±0.6	148±4	16.8	4.1±0.1	75.6±0.6	11.2±0.4	1.5±0.1
Barley	583	91±9	84.4±1.5	2665	426±42	84.0±1.6	59±3	10.6	3.8±0.4	64.1±3.8	3.8±0.2	3.0±0.4

* Number of observations for the low-protein hay 1, low-protein hay 2, high-protein hay and barley diets, were 5, 5, 16 and 9 respectively.

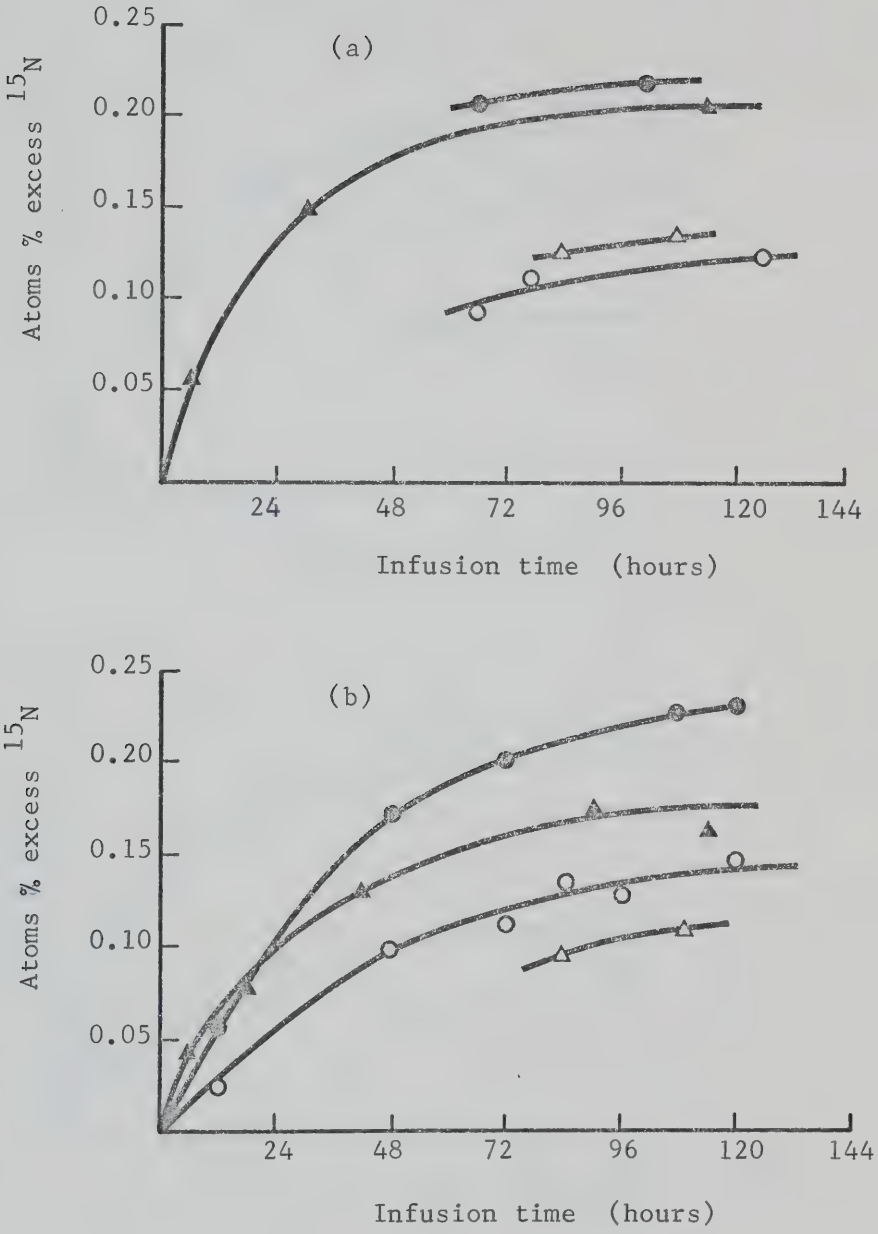

Figure 1. Incorporation of nitrogen from intraruminally infused $^{15}NH_4Cl$ into bacterial protein (●), protozoal protein (○), urinary nitrogen (▲), and fecal nitrogen (△) in samples taken from sheep given (a) low-protein hay 1 or (b) low-protein hay 2.

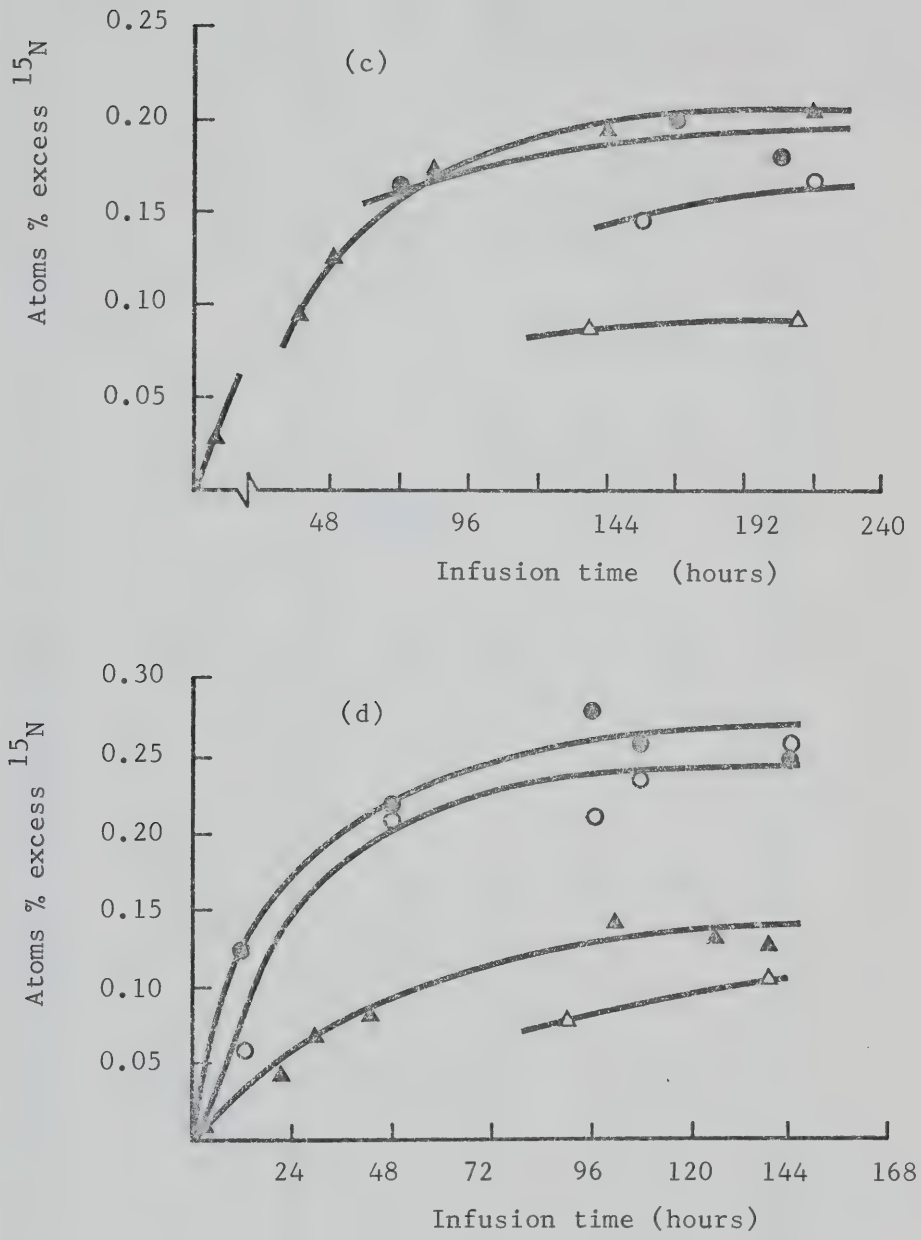

Figure 1. (cont'd). Incorporation of nitrogen from intraruminally infused $^{15}NH_4Cl$ into bacterial protein (●), protozoal protein (○), urinary nitrogen (▲), and fecal nitrogen (△) in samples taken from sheep given (c) high-protein hay or (d) barley. The break in the time axis of the graph (c) represents a 8 hour pause in infusion.

Table 2. Enrichments of ^{15}N in microbial, urinary and fecal nitrogen as a percentage of the concentration of excess ^{15}N in rumen ammonia at steady state.

Diet	Total bacterial	Bacterial protein	Total protozoal	Protozoal protein	Urinary	Fecal
Low-protein hay 1	--	55	--	31	53	34
Low-protein hay 2	--	65	--	40	50	31
High-protein hay	53	50	37	40	53	24
Barley	61	57	53	55	30	23

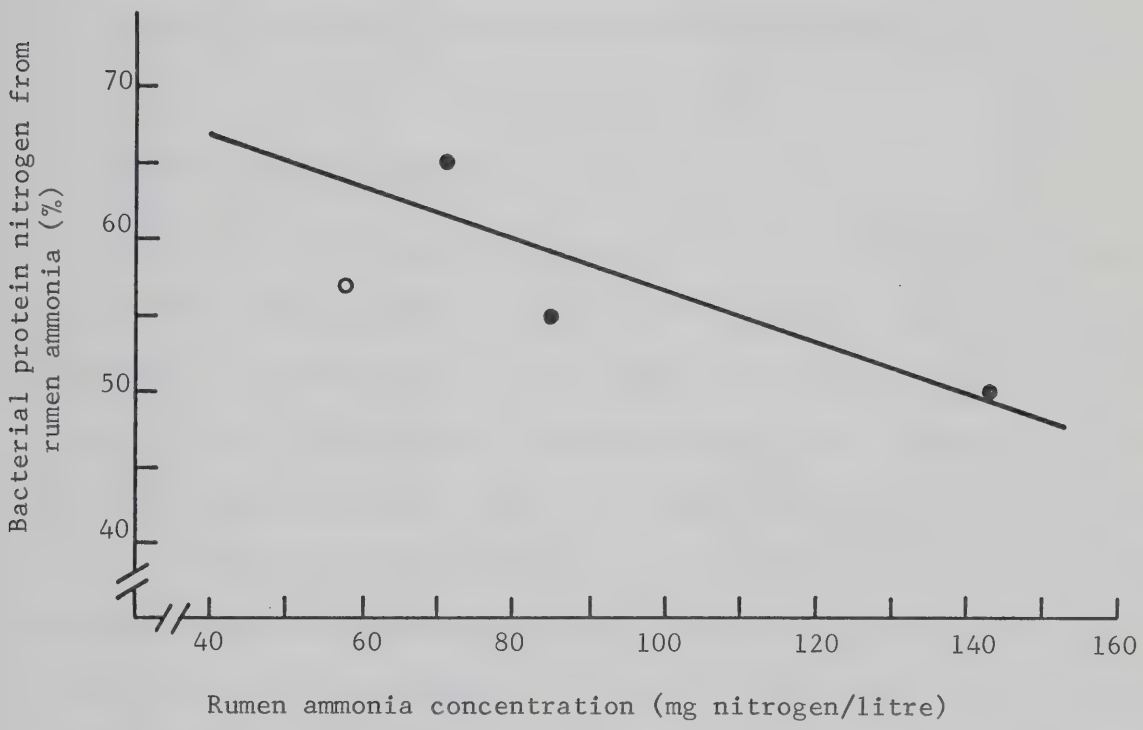

Figure 2. Relationship of percentage utilization of ammonia nitrogen by bacteria for cell synthesis and rumen ammonia concentration for sheep given hay diets. For comparison, the value for the sheep given the barley diet is also shown (o). The regression line is for the data on hay diets.

protein nitrogen and a slightly smaller percentage of total protozoal nitrogen (Table 2). The pathway for this incorporation could have been either directly through the utilization of ammonia nitrogen, or indirectly through the utilization of bacterial nitrogen. The enrichment of the protozoal fraction with ^{15}N was 56 to 96% of that of the bacterial fraction. Ulbrich and Scholz (1966a) obtained a corresponding value of 76% when ^{15}N-urea was given to cows and a more complete, but lengthy, method of protozoal separation that may have allowed metabolism during isolation was used.

B. Turn-over time of bacteria

The turn-over, or generation, time of the bacteria was calculated by first-order kinetic analysis (Sheppard, 1962) of the rate of enrichment of bacterial nitrogen with ^{15}N from ammonia as shown in Figure 3. The turn-over times for bacterial protein nitrogen in the rumen of the sheep given the low-protein hay 2 and barley diets were 42 and 38 hours respectively. Ulbrich and Scholz (1963) and Ulbrich and Scholz (1966a) reported turn-over times for bacteria in cattle of 27 and 28 to 29 hours. The times required for replacement (turn-over) of the rumen liquid before the trials were calculated from first-order analysis of the rate of disappearance of PEG from the rumen of the sheep given either the high-protein hay or barley diets, and values of 12.4 and 14.8 hours, respectively, were obtained (Figure 4). These turn-over times indicate that the bacteria in the rumen move predominately with the solid fraction of the rumen contents, and not with the liquid fraction.

C. Microbial growth

The amount of microbial nitrogen which passed through the abomasum

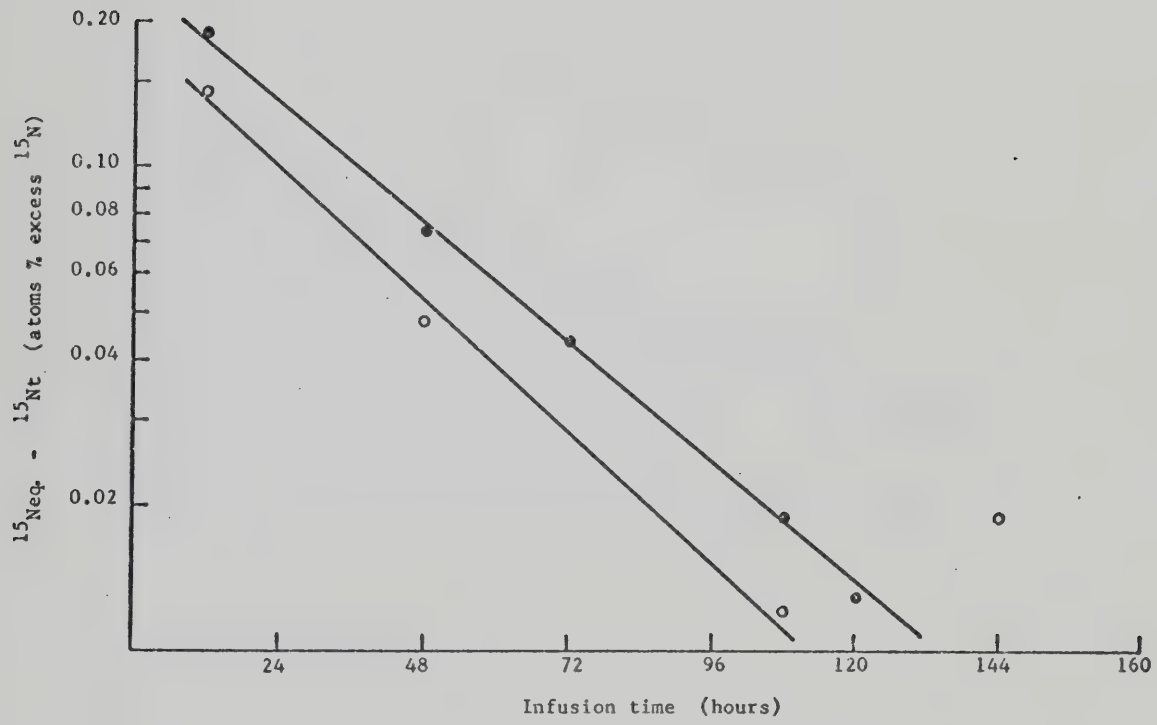

Figure 3. Rate of replacement of ^{14}N in bacterial protein nitrogen with intraruminally infused ^{15}N from $^{15}NH_4Cl$ in sheep given a low-protein hay diet (•), or a barley diet (o). $^{15}N_{eq.}$ refers to the concentration of isotope in the bacteria at steady state and $^{15}N_t$ refers to the concentration of ^{15}N at time t.

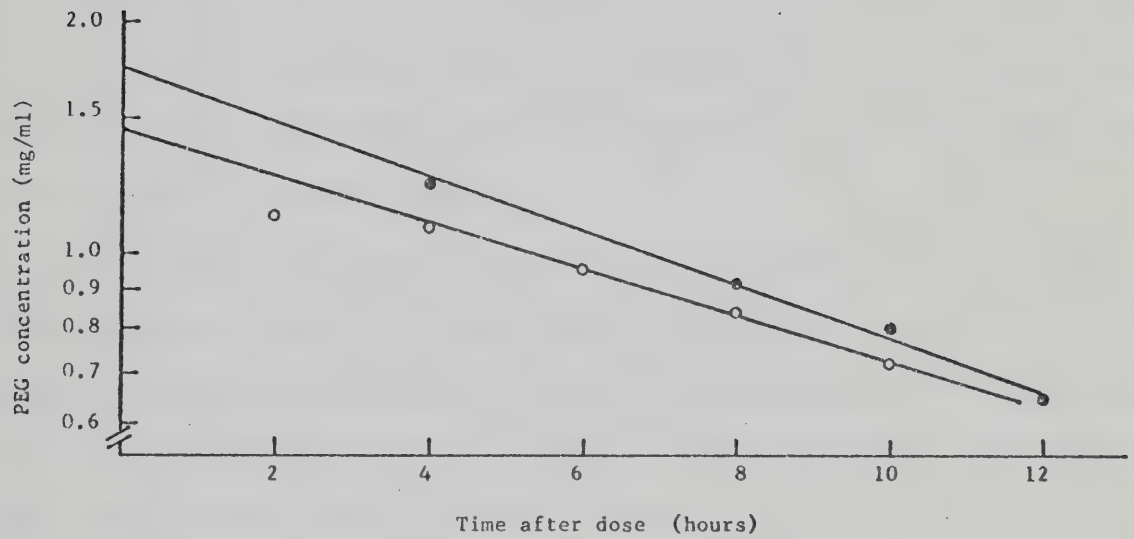

Figure 4. Rate of disappearance of polyethylene glycol (PEG) from the rumen of sheep given high-protein hay (●) or barley (o). The initial point on the barley line was considered to represent inadequate mixing.

daily was found to be from 7.8 to 9.4 g and from 9.2 to 12.9 g in the sheep given the high-protein hay and barley diets respectively (Tables 3 and 4). The larger value for each diet was calculated on the assumption that the soluble nitrogen passing through the abomasum, after correction for gastric juice nitrogen (1 g of nitrogen per hours; Weston and Hogan, 1967) and for rumen ammonia, was of microbial origin. Since there is considerable disruption of microbial cells in the abomasum, as evidence by microscopic observations (Annison and Lewis, 1959), and since most of the plant nitrogen is digested in the rumen (Weller, Pilgrim and Gray, 1962), the higher value is probably closer to the actual growth which took place in the rumen.

The barley diet supported the synthesis of from 1.1 to 1.3 times more microbial cellular nitrogen than the hay diet as measured by g of nitrogen assimilated per 100 g of dry matter fermented (Table 4). Yields of microbial nitrogen per unit of food energy fermented in the rumen from the two substrates, were, however, similar.

D. Production of rumen ammonia

Values for the metabolism of rumen ammonia are given in Table 5. The quantity of nitrogen converted into rumen ammonia was estimated from the extent of dilution of the infused isotope in rumen ammonia upon achievement of steady-state enrichment, as described by Steele et al. (1956). The calculation used was

$$\text{N converted into rumen ammonia (g N/day)} = \frac{^{15}\text{N infused (atoms/day)} \times 14 \text{ g N/atom}}{\text{steady-state enrichment of rumen ammonia (atoms \% excess } ^{15}\text{N)}}$$

The nitrogen converted into ammonia was equivalent to 60 to 92% of the

Table 3. Flow of digesta through the digestive tract of sheep given either barley or high-protein hay.

(Abomasal digesta were separated into various fractions as described on p. 10. Mean values with their standard errors for daily samples for each sheep; numbers of observations in parentheses).

Diet	Intake	Digesta passing through the abomasum				Proportions of plant components fermented in rumen (%)
		Total	Plant	Particulate microbial*	Soluble+	
			Dry Matter (g/day)			
Barley	583	223 ± 11(11)	87 ± 4(4)	136	-	85.1 ± 0.7(4)
High-protein hay	667	298 ± 7(15)	200 ± 4(5)	98	-	70.0 ± 0.6(5)
			Nitrogen (g/day)			
Barley	10.6	17.4 ± 0.7(11)	3.0 ± 0.2(4)	9.2	5.2 ± 0.2(10)	71.7 ± 1.9(4)
High-protein hay	16.8	15.8 ± 0.3(15)	3.5 ± 0.2(5)	7.8	4.5 ± 0.1(9)	79.2 ± 1.2(5)
			Gross Energy (kcal/day)			
Barley	2665	1016 ± 62(11)	425 ± 23(4)	591	-	84.0 ± 0.9(4)
High-protein hay	2868	1345 ± 28(15)	1056 ± 27(5)	289	-	63.2 ± 0.9(5)

* Particulate microbial = total - plant.
\+ Includes ammonia N.

Table 4. Microbial growth in the rumen of sheep given either barley or high protein hay.

Measure of growth	Barley		High-protein hay	
	a*	b+	a*	b+
Total nitrogen incorporated (g/day)	9.2	12.9	7.8	9.4
Nitrogen assimilated / 100 g DM ≠ fermented (g)	1.9	2.6	1.7	2.0
Nitrogen assimilated / 1000 kcal energy fermented (g)	4.1	5.8	4.3	5.2
Yield of cell wt / 100 g DM fermented, assuming 10.5% nitrogen in cells (g)	18.1	24.8	16.2	19.0

* Calculated on the assumption that the soluble, non-ammonia nitrogen passing through the abomasum was not of microbial origin.

\+ Calculated on the assumption that the soluble, non-ammonia nitrogen passing through the abomasum, corrected for digestive secretions as described on p. 22, was of microbial origin.

≠ Dry matter

Table 5. Metabolism of rumen ammonia in sheep given either barley or hay.

(Mean values; their standard errors given where appropriate; numbers of observations in parenthesis)

	Low-protein hay 1	Low-protein hay 2	High-protein hay	Barley
Ammonia concentration (mg nitrogen/litre)	85±4(6)	71±5(6)	143±3(7)	58±5(6)
Quantity of ammonia in rumen (mg nitrogen)*	-	-	815	406
^{15}N infused (mg ^{15}N/day)	43.27	47.50	42.60	41.84
Atoms % excess ^{15}N in rumen ammonia	0.391±0.007(3)	0.356±0.012(4)	0.394±0.018(5)	0.460±0.033(5)
Conversion of nitrogen into rumen ammonia (g nitrogen/day)+	10.34	12.46	10.10	8.48
Turn-over time of ammonia based upon conversion of nitrogen into ammonia (min.)≠	-	-	116	69
Urea recycled to rumen (g nitrogen/day)	-	-	6.0	3.0
Possible conversion of nitrogen into ammonia (g nitrogen/day)S	-	-	13.28	9.38
Possible turn-over time of ammonia (min.)Y	-	-	88	62

* Ammonia concentration (mg nitrogen/litre) x rumen volume (litres).
\+ Calculated from extent of dilution of infused ^{15}N in ammonia nitrogen.
≠ Quantity of ammonia in rumen (mg nitrogen)÷conversion of nitrogen into ammonia (mg nitrogen/min).
S Conversion of nitrogen into rumen ammonia (g nitrogen/day)+that portion of recycled urea nitrogen that could have had a concentration of ^{15}N equal to that of ammonia nitrogen.
Y Quantity of ammonia in rumen (mg nitrogen)÷possible conversion of nitrogen into ammonia (mg nitrogen/min).

daily nitrogen intake. Increases in the concentration of rumen ammonia in sheep given the hay diets were accompanied by decreases in the conversion of nitrogen into ammonia when this conversion was expressed as a percentage of total nitrogen intake (Figure 5). This relationship was described by the equation $Y = 123 - 0.44X$, where Y = nitrogen converted into rumen ammonia, expressed as a percentage of nitrogen intake, and X = concentration of rumen ammonia (mg nitrogen/litre).

Pilgrim, Gray and Belling (1969) found that the production of rumen ammonia was equivalent to 25% of the daily nitrogen intake in sheep given a hay-chaff of very high protein content and with rumen ammonia concentrations of 201 mg per litre. The equation derived above can be used with the concentration of ammonia in the rumen reported by Pilgrim, Gray and Belling (1969) to obtain a predicted value of 35%, which is quite similar to the value obtained by these investigators.

It is possible that more nitrogen was converted into ammonia than the quantity measured would indicate. This greater quantity, referred to as the possible conversion of nitrogen into ammonia in Table 6, would be dependent upon the amount of urea that was recycled to the rumen from the blood and the enrichment of the recycled urea. The value of 3 g of nitrogen for the amount recycled as urea in the sheep given the barley diet was estimated from an equation derived by Ford (1969) for sheep under the same management and feeding conditions as used in this study. This equation relates urea recycling to plasma urea concentration. The urea recycled was predicted for a plasma urea concentration of 15 mg of urea per 100 ml, which was the concentration measured in this sheep. The plasma urea concentration of 71 mg urea per 100 ml of plasma measured for the sheep given the high-protein hay diet was sufficiently high to

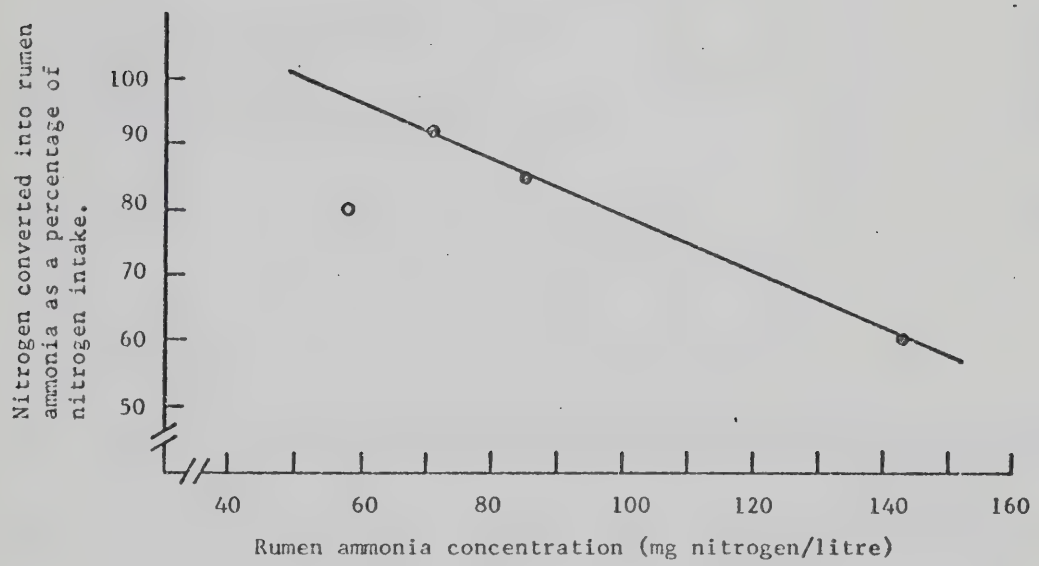

Figure 5. Relationship of the quantity of nitrogen converted into rumen ammonia, expressed as a percentage of daily nitrogen intake, and rumen ammonia concentration for sheep given hay diets (•). The result for the barley diet is also shown (o) for comparison.

Table 6. Quantitative estimates of the metabolism of nitrogen in the rumen of sheep given either barley or high-protein hay

Rumen N* flux	Hay	Barley
Inflow of rumen N (g/day):		
Food N	16.8	10.6
Urea N recycled	6.0	3.0
Unaccounted for N**	---	4.4
Total inflow of rumen N (g/day)	22.8	18.0
Outflow of rumen N (g/day):		
Microbial N from ammonia +	4.2	7.3
Rumen ammonia passing abomasum	1.9	0.5
Ammonia absorbed from rumen#	7.2	1.6
Undigested food N	3.5	3.0
Microbial N directly from food ≠	5.2	5.6
Absorbed amino acid N**	0.8	---
Total outflow of rumen N (g/day)	22.8	18.0

* Nitrogen

** Difference between measured total N inflow and total N outflow.

\+ Proportion of microbial N from ammonia (average of bacterial N and protozoal N, Table 2) x maximum microbial incorporation of N (Table 4).

\# Possible conversion of N into ammonia (Table 5) - (Microbial N from ammonia + rumen ammonia passing abomasum).

≠ Proportion of microbial N not from ammonia (average of bacterial N and protozoal N, Table 2) x maximum microbial incorporation of N (Table 4).

presume that there was maximal recycling of urea, which would be 6 g of nitrogen daily in sheep (Ford, 1969; Cocimano and Leng, 1967; Weston and Hogan, 1967). The ^{15}N enrichment of the recycled urea nitrogen was assumed to be similar to the enrichment of the total urinary nitrogen, since Ulbrich and Scholz (1966b) reported that urinary urea nitrogen was only slightly more enriched than total urinary nitrogen after the oral intake of ^{15}N-urea by cows.

E. Absorption from the rumen

Quantitative estimates of metabolism of nitrogen in the rumen are given in Table 6. Microbial estimates include that portion of the soluble fraction which was assumed to have arisen from microbial disintegration in the abomasum, which, as indicated previously, is the soluble nitrogen passing through the abomasum corrected for nitrogen in the ammonia and digestive secretions.

A considerable amount of ammonia nitrogen was absorbed from the rumen, which is in agreement with reports of other workers (Gray, Pilgrim and Weller, 1958). Pilgrim, Gray and Belling (1969) found that from 3 to 4.6 g of ammonia nitrogen were absorbed every day from the rumen of a sheep given a high-protein hay.

It is suggested that 0.8 g of nitrogen was absorbed from the rumen in the form of amino acids or other non-ammonia nitrogen when the high-protein hay ration was fed; such absorption would account for the difference between the flow of nitrogen into and out of the rumen (Table 6). The absorption of amino acids from the rumen has been demonstrated previously by Cook, Brown and Davis (1965).

DISCUSSION

A. Microbial utilization of rumen ammonia

The large percentage utilization of ammonia for bacterial synthesis in the present experiments (Table 2) agrees with *in vivo* results reported previously. Portugal (1963), using carbon-labelled amino acids found that only about 10% of the carbon of amino acids is incorporated into microbial protein in the rumen, while Weller, Pilgrim and Gray (1962) concluded that up to 80% of the dietary plant nitrogen is incorporated into microbial cells. These findings indicate that the carbon and nitrogen of dietary protein are separated during metabolism in the rumen before incorporation of the nitrogen into microbial cells. Bryant and Robinson (1962) have shown both a requirement and a preference by certain rumen organisms for ammonia compared with amino acids. Hobson, McDougall and Summers (1968) found that one rumen isolate formed 93% of its cellular nitrogen from ammonia. Clearly, rumen ammonia is a very important source of rumen microbial nitrogen.

Investigations using heterotrophic bacteria have suggested that there is tendency towards increased incorporation of amino acids and peptides when these are available (Roberts *et al*. 1955; Warner, 1956). Since there is a positive correlation between the concentrations of non-protein, non-ammonia nitrogen and ammonia nitrogen in the rumen (Blackburn and Hobson, 1960b), when concentrations of ammonia increase there are likely to be enhanced amounts of amino acids and peptides available to the rumen population. This would support the suggestion, from the admittedly small number of results in the experiments, that percentage utilization of ammonia by bacteria decreased as concentrations of rumen ammonia increased when the hay diets were given (Figure 2). This also

explains the negative correlation between the nitrogen converted into rumen ammonia as a percentage of nitrogen intake and the rumen ammonia concentration (Figure 5).

The concentration of isotope in the total nitrogen content of the bacteria was slightly higher than that in the protein nitrogen fraction (Table 2). This would indicate that incorporated non-protein nitrogen is a precursor of protein nitrogen in bacterial cells. It is also possible that the proportion of the nitrogen in nucleic acids that is derived from ammonia is greater than that in protein. The large concentrations of nucleic acids in rumen microorganisms (Smith, McAllan and Hill, 1968) indicate substantial synthesis of these acids in the rumen.

B. Microbial growth

The microbial yields of 1.7 to 2.6 g of nitrogen assimilated per 100 g of dry matter fermented in the rumen obtained in these experiments are considerably higher than the estimate of 1.1 g of nitrogen incorporated per 100 g of dry matter fermented derived by Hungate (1966). Microbial yields in the range of those obtained in this experiment (2 g of nitrogen assimilated for every 100 g of substrate fermented) have however, been obtained by various workers under in vitro conditions of continuous culture (Hungate, 1963; Hobson, 1965; Hobson and Summers, 1967). Walker and Nader (1970) estimated that the amount of nitrogen assimilated by rumen microorganisms was equivalent to 2.3% of the substrate fermented from in vitro results on the incorporation of radioactive sulfide.

Few in vivo results have been reported concerning the yield of microbial cells per unit of substrate fermented in the rumen. Conrad and Hibbs (1968), from in vivo studies in which methionine synthesized in

the rumen was used as an indicator of the total cell nitrogen synthesized, estimated that 1.63 g of nitrogen per 100 g of digestible dry matter were assimilated in the cow's rumen. Hume (1970) measured a very high cell yield of 3.7 g of nitrogen assimilated for every 100 g of dry matter fermented in the rumen of sheep fed a semi-purified diet.

Results reporting a relatively high yield of microbial cells in the rumen, such as those reported above, led Purser (1970) to conclude that microorganisms incorporate nitrogen into cellular material in amounts greater than 1.1% of the fermented material, which was the value suggested by Hungate (1966). This is in agreement with the results obtained in this experiment where it was found that rumen microorganisms were almost twice as efficient in incorporating nitrogen into cellular materials as the estimates of Hungate (1966) would suggest.

The yield of microbial cellular nitrogen can also be estimated from the flow of digesta through the abomasum and proximal duodenum. This flow was measured by Meyer et al. (1959), Hogan and Phillipson (1960), Harris and Phillipson (1962), Hogan and Weston (1967a, b, 1969a), Weston and Hogan (1968) and Topps, Kay and Goddall (1968), and their results were used to relate the net loss of nitrogen in the rumen to the percentage nitrogen in the food. The equation obtained was: $Y = 4.31 X - 8.61$ where Y = difference between the intake of nitrogen in the food (g per day) and the passage of nitrogen through the abomasum or duodenum (g per day) and X = percentage of nitrogen in the dry matter of the food. The standard error of the estimate was 2.40 g of nitrogen and the correlation coefficient was 0.886. In deriving this equation, it was assumed that 1 g of gastric juice nitrogen was added to the abomasum daily (Weston and Hogan, 1967). Then, Y = 0 when the diet

contains 2% nitrogen. It may be assumed that any large excess of rumen ammonia passing from the rumen is absorbed in the omasum, as indicated by results of Hogan and Weston (1967a), and that the extent of digestion of dry matter and of nitrogen are similar. This equation would then suggest that there was assimilation of nitrogen by the microbes to the extent of 2% of the fermentable substrate in the rumen; that is, for every 100 g of dietary dry matter fermented in the rumen, 2 g of dietary nitrogen have been digested and 2 g of nitrogen pass into the abomasum in cell material. Hobson (1965) and Hobson and Summers (1967) have shown that the yield of microbial cellular material in anaerobic fermentation is directly proportional to the growth rate of the microbes. The apparently large yields of 1.7 to 2.6 g of nitrogen assimilated into cell material by the microorganisms per 100 g of dry matter fermented appear to be inconsistent with the slow turn-over time of approximately 40 hours found for the bacterial cells. There are many instances of changes in the enzymic complement of bacteria in response to different conditions. It is possible that organisms in the rumen adapt to conditions in which substrate is continuously available, and the removal of end-products is rapid, by reducing endogenous metabolism and by using substrates more efficiently.

C. Utilization of absorbed rumen ammonia

There was considerable absorption of ammonia from the rumen (Table 6). Ammonia is converted into urea in the liver (Lewis, Hill and Annison, 1957); thus, if the ammonia that left the rumen through the rumen wall was transported as such to the liver and mixed with the ammonia serving for urea synthesis in the present experiments, it should have contributed to the ^{15}N enrichment of urea within the relatively

short time that was required for the blood to flow from the rumen to the liver. Ford (1969) found that, after intravenous injections of ^{14}C-urea in sheep, 85% of the ^{14}C that was excreted in the urine appeared within 12 hours. Figure 1 shows that the increase in enrichment of urinary nitrogen was not rapid during infusion of $^{15}NH_4Cl$, even though the enrichment of rumen ammonia nitrogen reached a steady state within 12 hours when the sheep was given the barley ration. Instead, the increase in enrichment of urinary nitrogen was continuous and similar to the increase of ^{15}N in the microbial nitrogen. Thus, absorbed ammonia may not have been converted directly into urea in substantial amounts in our sheep. Studies in which ^{15}N-labelled ammonium sources were provided to the rumen of cows have shown a rapid incorporation of ammonia nitrogen into both total nitrogen and protein nitrogen in milk (Land and Virtanen, 1959; Faust et al., 1963 ; Faust et al., 1966), which also indicates that absorbed ammonia nitrogen may be utilized by a pathway other than the direct conversion into urea.

Glutamate dehydrogenase has been found in rumen epithelium, and the formation of glutamate from 2-oxoglutarate may occur in the rumen epithelium as well as the formation of glutamine from glutamate (Hoshino, Sarumaru and Morimoto, 1966). Fixation of ammonia nitrogen in rumen epithelium would protect the animal tissues from the toxic effects of ammonia and would enable the ruminant animal to control the rate of formation of blood urea from rumen ammonia. This would reduce the recycling of urea, a process which may waste energy through ureolysis and eventual synthesis of urea from the resultant ammonia. The fixation of ammonia nitrogen could also be important as an extrahepatic site for the synthesis of amino acids and could facilitate transport of rumen

ammonia to other extrahepatic sites of amino acid synthesis. At high concentrations of rumen ammonia, the process of fixation in the rumen wall may become saturated so that a rise in portal blood ammonia would occur, as reported by Lewis, Hill and Annison (1957).

Schoenheimer, Ratner and Rittenberg (1939) obtained results with rats that indicated a rapid exchange of glutamic acid nitrogen with food and body amino acid nitrogen. The retention of labelled nitrogen within the tissues of the sheep and to some degree the delay in excretion of ^{15}N in the urine observed may not only indicate the extent of net utilization of ammonia, but could reflect exchange within the animal body as well.

D. Sources of rumen nitrogen in sheep fed low protein diets

Approximately 4 g of nitrogen appeared to enter the rumen of the sheep given the barley ration which were not accounted for by normal ways of entry (Table 6). It is fully recognized that this result was derived in only one trial and must be considered with reservation. However, errors of the order of 47, or 51%, in the estimates of nitrogen outflow from rumen ammonia, or of outflow of nitrogen that was not converted into ammonia, respectively, would have been necessary to equalize inflow and outflow. In addition, results obtained by Topps, Kay and Goodall (1968), Harris and Phillipson (1962) and Weston and Hogan (1968) all show that there was net addition of large amounts of nitrogen (up to 6 to 7 g/day) to the rumen when sheep consumed diets containing low levels of nitrogen (1 to 1.5% nitrogen). It is unlikely that this major source of nitrogen entering the rumen when low protein diets were fed can be explained entirely by urea recycling since a maximum of 4 to 6 g of urea nitrogen are recycled to the rumen each

day (Weston and Hogan, 1967; Cocimano and Leng, 1967; Ford, 1969), and this amount is considerably less when the diet has a low protein content.

This would suggest that there may, under some circumstances, be another major source of entry of nitrogen into the rumen in addition to feed and urea nitrogen. It is possible that this nitrogen arose from the fixation of gaseous nitrogen in the rumen, since the conditions of low rumen ammonia content and a highly fermentable substrate would be expected to favor such a process. If gaseous nitrogen was fixed in the rumen, it may have been converted into rumen ammonia as shown (Table 6), or it may have been fixed by the microbes without equilibration with extracellular ammonia, which, by our method of calculation, would cause an underestimation of the conversion of food nitrogen into ammonia in this sheep.

Hungate (1966) has suggested that under usual ruminal conditions there would be adequate fixed nitrogen for microbial growth, but under certain conditions there might be selection for a type of bacteria which would fix nitrogen gas. The possibility of nitrogen gas fixation will be examined in Section II of this thesis.

Compounds other than urea may also enter the rumen from the blood and thus contribute to rumen nitrogen. Leibholz (1971a) observed an apparent transfer of amino acids into the rumen of sheep and it is possible that some similar process may also be responsible for the additional nitrogen which entered the rumen nitrogen pool in the sheep fed the barley diet. This possibility will be examined in Section IV of this thesis.

SUMMARY

$^{15}NH_4Cl$ was continuously infused into the rumens of sheep which were allowed to feed 2 out of every 10 minutes for periods of up to 9 days. The sheep were given either a barley diet or one of three hay diets. These treatments achieved steady metabolic states and allowed the assessment of nitrogen conversions by means of tracer methodology. In two trials the flow of abomasal material was determined using lignin and polyethylene glycol as markers, and from these results and the ^{15}N concentration of abomasal material, the yield of microbial cells and the extent of degradation of plant material in the rumen were determined.

Results obtained by comparison of the steady-state concentration of ^{15}N in the microbes with that in rumen ammonia indicated that from 50 to 65% of the bacterial nitrogen and from 31 to 55% of the protozoal nitrogen were derived from rumen ammonia *in vivo*.

The extent of dilution of the ^{15}N-labelled ammonia in the rumen was used to calculate the entry rate of ammonia into the rumen ammonia pool, and it was determined that an amount of nitrogen equivalent to 60 to 92% of the daily intake was transformed into ammonia nitrogen in the rumen. Approximately 17 to 54% of the ammonia was absorbed from the rumen, but this ammonia did not appear to be readily converted to urea.

Microbial growth in the rumen was studied and it was found that microbial cells assimilated 1.7 to 2.6 g of nitrogen for every 100 g of dry matter fermented in the rumen. In addition, the generation-time of bacterial protein was calculated from the rate of increase of ^{15}N concentration in this fraction, and values of 38 and 42 hours were obtained for sheep given barley and hay diets respectively.

The need for further studies into rumen nitrogen metabolism was shown from an assessment of the information in the literature on this subject and the information derived in the present experiment.

NOTE

After the experiments described in this Section were completed and submitted for publication, at least two similar experiments have appeared concerning the utilization of ammonia nitrogen by rumen microorganisms and the growth of microorganisms in the rumen.

Pilgrim et al. (1970) carried out experiments essentially similar to those reported in this Section using $(^{15}NH_4)_2SO_4$ to label rumen ammonia. They obtained very similar results to those presented here with respect to the utilization of ammonia for microbial synthesis and estimates of cell yield. They found that from 62 to 78% of the bacterial nitrogen was formed from rumen ammonia and that 35 to 64% of the protozoal nitrogen arose from this source. They also reported a tendency for decreased incorporation of ammonia into microbes when the nitrogen content of the diet increased.

Measurements of ammonia production made by Pilgrim et al. (1970) were similar to those reported here. In addition similar results were obtained for the amount of microbial nitrogen formed in the rumen. These workers assumed that the difference between ammonia production in the rumen and the amount of ammonia leaving the rumen by absorption or by passage into the omasum was equal to microbial incorporation of ammonia nitrogen and thus calculated that a maximum of 8.5 to 12.5 g of microbial nitrogen were synthesized daily in the rumen of the sheep fed 800 g of hay chaff.

These workers gave no values for the turn-over time of bacterial

nitrogen in the rumen, or for the enrichment of the urine and feces with ^{15}N. Also no measurement was made of the extent of plant degradation in the rumen.

Recently Al-Rabbat, Baldwin and Weir (1971) reported results obtained by incubating rumen contents with ^{15}N *in vitro*. From a measurement of the incorporation of ^{15}N-ammonia into microorganisms and an estimation of microbial efficiency, it was suggested that from 45 to 64% of rumen microbial nitrogen was derived from ammonia and that the yield of microbial nitrogen in the rumen would be 1.6 to 2.2 g of nitrogen assimilated per 100 g of digestible organic matter.

Both of the reports cited above provided results which were very similar to those presented in Section I of this thesis. These experiments, as well as confirming the results obtained in this Section, also indicate the importance which various workers placed upon the determination of the proportion of microbial nitrogenous compounds synthesized from rumen ammonia and the yield of microbial material in the rumen.

SECTION II

THE FIXATION OF NITROGEN GAS IN THE RUMEN

INTRODUCTION

In Section I of this study on the nitrogen metabolism of the ruminant animal the possibility of nitrogen gas fixation in the rumen was invoked to explain the entry of nitrogen into the rumen which was not accounted for by the measurements made. Hobson, McDougall and Summers (1968) have also indicated that there is some evidence that nitrogen gas fixation may occur in the rumen. In addition, bacteria of the Methanobacterium and Clostridium species are present in the rumen (Hungate, 1966) and are potentially capable of fixing nitrogen gas.

Ruminal conditions are both favorable and unfavorable for the process of nitrogen gas fixation. The near neutral pH, the highly reducing conditions and the presence of a readily available energy source when carbohydrates are fed, would be conducive to the process of nitrogen fixation. Conversely, the relatively high levels of ammonia which exist in the rumen under normal conditions would tend to inhibit the formation of the nitrogen fixing enzyme system (Dalton and Postgate, 1969).

At the time this experiment was started, no reports were found in the literature on any isotopic studies which had been conducted to study nitrogen fixation by rumen microorganisms. Since any fixation of nitrogen gas in the rumen could be extremely important in the nitrogen metabolism of the ruminant animal and in the interpretation of experimental results, this experiment was designed to test for the possibility of the incorporation of inorganic nitrogen gas into an organic fraction by rumen microorganisms.

LITERATURE REVIEW

A. Biological nitrogen fixation

Nitrogen fixation enables organisms to live and grow in conditions where available organic nitrogen would limit the growth of the organism. A variety of organisms can fix nitrogen into an organic form under both aerobic and anaerobic conditions. Examples of potential nitrogen fixing bacteria which are found in the rumen are bacteria of the Methanobacterium and Clostridium species (Hungate, 1966).

Nitrogen gas fixation is characterized by the reduction of gaseous nitrogen to ammonia. No stable or transient intermediate has been isolated in this conversion (Postgate, 1970a). Since the process is endergonic and reductive in nature, both energy and a source of electrons are required. Dalton and Postgate (1969) have suggested that from 4 to 5 moles of ATP are required for every mole of N_2 fixed. It is possible that pyruvate, a normal rumen microbial metabolite (Hungate, 1966), could be utilized as an electron and energy source for this process in the rumen, since Carnahan et al. (1960) have shown that pyruvate can supply the reducing power and the energy for nitrogen fixation in Clostridium pasteurianum.

Since N_2 fixation is an energetically costly process, its occurrence may be unlikely when adequate organic nitrogen is available for microbial growth. The formation of the enzyme system responsible for nitrogen fixation, has in fact, been found to be sensitive to the presence of organic nitrogen. Dalton and Postgate (1969) stated that ammonia inhibited the formation of the nitrogen fixing enzyme system and thus the existence of this system in the rumen under many conditions may be improbable.

It has been shown that nitrogen fixation is also inhibited by oxygen

in facultative anaerobes (Pengra and Wilson, 1958). Oxygen can react with the labile sulfide groups in the enzyme system rendering it inactive (Postgate, 1970a). This characteristic of the enzyme system, makes its detection technically difficult.

B. The detection of nitrogen fixation

Three different methods have been utilized for the detection of nitrogen fixation: these include nitrogen balance studies, the detection of nitrogenase utilizing the acetylene reduction test, and the measurement of the incorporation of gaseous isotopic nitrogen into an organic form.

Nitrogen balance studies to detect nitrogen fixation involve the measurement by the Kjeldahl, or other suitable method, of an increase in the total organic nitrogen of a biological system exposed to nitrogen gas. This procedure will not detect increases in nitrogen of less than 1% of the total organic nitrogen present in the system, thus it is only useful for active nitrogen fixers (Burris and Wilson, 1957).

The nitrogen fixing system, as well as reducing nitrogen gas to ammonia, also reduces the bonds in acetylene, cyanides, azides, nitrous oxide and related compounds (Postgate, 1970a). Since ethylene, the reduced product of acetylene, can be detected in extremely small amounts by gas chromotography, the acetylene reduction test has often been used to establish the existence of the enzyme complex. Recently, however, it has been shown that there is a small reduction of acetylene when acetylene and hydrogen are shaken with suspended particles such as bacterial cells that do not have nitrogen fixing activity (Hobson, 1969). The possibility of such a non-enzymatic reaction occurring limits the usefulness of this technique in establishing the existence of the

nitrogen fixing enzymes.

Probably the best method of detecting the fixation of nitrogen is by incubating the test organism with isotopic nitrogen gas. Any incorporation of isotope into an organic form is taken as evidence of nitrogen gas fixation. The problem with this method of nitrogen fixation detection is the lack of sensitivity. Burris and Wilson (1957) claimed that enough isotopic nitrogen must be incorporated into a sample to enrich the nitrogen to 0.015 atoms % excess ^{15}N in order to be measured with a mass spectrometer with sufficient accuracy to prove nitrogen gas fixation. In spite of the lack of sensitivity and the high cost of the $^{15}N_2$ method, it is undoubtedly the one of choice for demonstrating the fixation of nitrogen gas.

C. Nitrogen fixation in the rumen

Hungate (1966) has suggested that under usual ruminal conditions there would be adequate fixed nitrogen for microbial growth, but under certain conditions there might be selection for a type of bacteria which would fix nitrogen gas. Hobson, McDougall and Summers (1968) found that up to 7% of the cellular nitrogen incorporated into Bacteroides amylophilus was not derived from any identified nitrogen source when this organism was incubated with ^{15}N-ammonia, and thus concluded nitrogen fixation may occur in this organism.

The acetylene reduction test has recently been used by some workers to demonstrate the existence of the nitrogen fixing enzyme system in organisms from the digestive tract of animals. Granhall and Ciszuk (1971) tested rumen microorganisms by this method and found that small amounts of ethylene were formed from acetylene. The authors related this formation to nitrogenase activity and concluded that a maximum of

1.1 mg of nitrogen per day could be fixed in the rumen of a sheep if the activity of the enzyme *in vivo* was similar to that determined *in vitro*. Bergersen and Hipsley (1970) found organisms in the feces of animals which had the ability to reduce acetylene as well as to incorporate $^{15}N_2$, and suggested that nitrogen fixing organisms were present in the digestive tract of monogastrics. A report by Hobson (1969) also demonstrated the conversion of small amounts of acetylene into ethylene by rumen microorganisms. It was found, however, that this conversion might have occurred by non-enzymatic means. This means that the demonstration of nitrogen fixing activity by the acetylene reduction test is subject to doubt, and thus any demonstration of nitrogen fixing ability by rumen microorganisms by this method does not prove that nitrogen fixation occurs in the rumen.

Nitrogen gas fixation has also been studied using $^{15}N_2$ gas. The rumen microbe *Bacteroides amylophilus* did not incorporate $^{15}N_2$ into an organic form when grown in an atmosphere containing the isotope (Hobson, 1969). Postgate (1970b), however, suggested that a sulfate - reducing microorganism, which is found in the rumen, was capable of incorporating $^{15}N_2$ gas. The level of enrichment attained after 19 days incubation was, however, only 0.01 atoms % excess ^{15}N. This level of enrichment is only barely measurable above background (Burris and Wilson, 1957), and thus this evidence of nitrogen fixation can be questioned.

The only *in vivo* $^{15}N_2$ gas fixation experiment with ruminants reported in the literature was conducted by Moisio, Kreula and Virtanen (1969). These workers could detect no enrichment of organic nitrogen in the rumen when $^{15}N_2$ gas was bubbled through the rumen of a cow, however, no information was given concerning the length of time involved in the

experiment. Further *in vivo* $^{15}N_2$ experiments by these workers were limited because of the expense of the isotope.

From these experimental results to date it can be concluded that suggestions of nitrogen fixation by rumen microbes are tenuous. These reports, however, do not rule out the possibility of N_2 fixation in the rumen because the negative results from the *in vitro* isotope experiments were obtained with rumen isolates and the conditions in the rumen in the *in vivo* experiment may not have been favorable for N_2 fixation.

EXPERIMENTAL

A. Methods

The possibility of nitrogen gas fixation by rumen microorganisms was studied by incubating either whole rumen contents, or washed cells, from the rumen with $^{15}N_2$ gas and measuring the ^{15}N concentration in the organic nitrogen fraction in an effort to detect any ^{15}N accumulation.

B. Incubation of whole rumen contents

Samples of rumen contents were obtained from the rumen of a sheep, or cow, through a rumen fistula with care being taken not to expose the sample to air. These samples were then flushed with CO_2 to maintain anaerobic conditions.

Approximately 100 g of the whole rumen contents were incubated with 10 to 20 ml of $^{15}N_2$ gas (99 atoms % ^{15}N, Bio-Rad Laboratories, Richmond California) for periods of 2 to 8 hours in a water bath at 38 C. All samples were isolated from the atmosphere during incubation and the gas containing the isotope was mixed with the rumen sample in a closed system through a sintered glass bubbler by a recirculating diaphragm pump or by mechanically shaking the sample in an Erlenmeyer flask.

C. Incubation of washed cell preparations

Two samples of washed rumen cells were prepared by centrifuging 60 ml of rumen liquid, obtained by filtering rumen contents through six layers of cheese-cloth, at 19000 x g for 20 minutes. These cells were then washed in a sterile solution of 0.85% (W/v) saline and incubated, with continuous shaking, under 10 ml of $^{15}N_2$ gas in the medium described by Briggins and Postgate (1969) in 125 ml Erlenmeyer flasks. The carbon source used in the medium was starch and cellobiose for the cells

obtained from the rumen of the sheep fed barley and the cow fed hay, respectively.

The pH of the incubated samples was monitored and maintained at 6.1 to 6.3 by adding 1 M $NaHCO_3$ as required. The incubation period was for 48 hours.

D. Preparation of incubated samples for ^{15}N analysis

Incubated samples were separated into total rumen liquid, total rumen solids, ammonia, non-ammonia soluble nitrogen, bacterial and protozoal fractions as described in Section I (see Appendix B). Isolated samples were analysed for ^{15}N by the procedure used in Section I and described in Appendix D.

RESULTS

The source of the incubated rumen microorganisms and the incubation conditions used are shown in Table 7. The results of the ^{15}N analysis in each experiment are also given as an average of the ^{15}N concentration in the various fractions isolated.

The results of the ^{15}N analysis reported in Table 7 show quite clearly that no measurable incorporation of $^{15}N_2$ occurred in these experiments. Since the possibility that some nitrogen fixation did occur and was not measured was substantially reduced by separating the incubated samples into various fractions before ^{15}N analysis it can be concluded that no nitrogen fixation occurred in these experiments.

Table 7. Incubation conditions and results of incubating rumen microorganisms with $^{15}N_2$.

Diet	Animal	Rumen ammonia concentration (mmoles/litre)	Weight of sample incubated (g)	$^{15}N_2$ added (mmoles)	Incubation time (hours)	Atoms % ^{15}N in blank	Average atoms % ^{15}N in isolated samples*	
				Whole rumen contents				
straw:hay	cow	--	114	0.38	4	0.358	0.358±0.003	(6)+
hay + grain	cow	15.2	110	0.57	2	0.363	0.359±0.004	(3)≠
poor hay	cow	13.2	150	0.56	3	0.360	0.360±0.000	(3)S
barley**	sheep	24.4	105	0.54	8	0.366	0.358±0.001	(3)S
barley**	sheep	24.4	93	0.74	5	0.359	0.358±0.002	(3)S
				Washed cells				
poor hay	cow	--	60	0.36	48	0.355	0.360±0.001	(2)Y
barley**	sheep	20.4	60	0.36	48	0.370	0.358±0.003	(2)Y

* Mean ± S.D. with numbers of observations in parenthesis.
** Barley offered every 10 minutes from a mechanical feeder
\+ Total strained rumen fluid nitrogen; total rumen solids nitrogen; non-ammonia soluble nitrogen; and ammonia, bacterial and protozoal nitrogen were isolated.
≠ Ammonia, bacterial and protozoal nitrogen were isolated.
S Total strained rumen fluid nitrogen, ammonia nitrogen and bacterial nitrogen were isolated.
Y Total soluble nitrogen and bacterial nitrogen were isolated.

DISCUSSION

In agreement with other workers who have used $^{15}N_2$ in an effort to detect nitrogen fixation in the rumen (Hobson, 1969; Moisio, Kreula and Virtanen, 1969), no evidence of such a process in rumen microorganisms was found in the present experiments. This lack of evidence for nitrogen fixation was obtained even though workers have reported the detection of the nitrogenase enzyme system in rumen contents by means of the acetylene reduction test (Hobson, 1969; Granhill and Ciszuk, 1971). This suggests that the reported conversion of acetylene to ethylene by rumen microorganisms (Granhall and Ciszuk, 1971) could have been a non-enzymatic process as pointed out by Hobson (1969), and that nitrogen gas fixation does not occur in the rumen under normal conditions.

The two main factors which would likely limit the growth of nitrogen fixing organisms in the rumen are that ammonia is usually present in the rumen in relatively high concentrations, which would likely result in the inhibition of nitrogenase formation and that the fixation of nitrogen gas is an energetically costly process (Dalton and Postgate, 1969).

SUMMARY

Evidence for nitrogen gas fixation in the rumen was sought by incubating rumen contents and washed cells with $^{15}N_2$ gas under anaerobic conditions. No nitrogen gas fixation was detected.

SECTION III

LIVER AMMONIA METABOLISM

INTRODUCTION

Ammonia is produced in the rumen and is absorbed from this organ and converted to urea in the liver (McDonald, 1948). This process can result in the loss of substantial amounts of nitrogen to the animal (Lewis, Hill and Annison, 1957). Absorbed ammonia may also, however, be metabolized to compounds other than urea in the animal body.

One site in the body where ammonia could be utilized is the liver. Fontenot (1971) observed that it would be logical that some absorbed ammonia would be utilized for amino acid synthesis. The increases in the level of the liver enzymes NAD- and NADP-glutamate dehydrogenase, which were measured by Chalupa *et al*. (1970b) when a diet containing urea was fed to sheep, would support such a concept. Results presented in Section I of this thesis, which show a delay in the excretion of ^{15}N in the urine when ^{15}N-labelled ammonia was infused into the rumen, also indicate the possibility of metabolism of ammonia to compounds other than urea in the liver.

Since there are biochemical, nutritional and energetic implications to the formation of non-urea compounds from ammonia in the liver, the present experiment was undertaken to determine the extent of any such utilization of ammonia.

LITERATURE REVIEW

A. The absorption of rumen ammonia

The absorption of ammonia from the rumen has been demonstrated by various methods. Weller, Pilgrim and Gray (1962) and Hogan and Weston (1969a) are among the workers who measured a net loss of nitrogen from the rumen of sheep and suggested absorption of ammonia through the rumen wall to explain the loss. McDonald (1948) estimated that up to 5 g of nitrogen were absorbed from the rumen of a sheep daily. Ammonia losses from the rumen have been reported when ^{15}N-ammonia was placed in the rumen (Pilgrim, Gray and Belling, 1969; Pilgrim et al., 1970). The use of the isotope enabled these workers to measure the production of ammonia in the rumen, and from these measurements it was found that from 2.8 to 9.5 g of ammonia nitrogen were lost from the rumen of their sheep daily. In Section I of this thesis rumen ammonia absorption was also measured using ^{15}N and it was found that from 1.6 to 7.2 g of ammonia nitrogen was absorbed daily from the rumen of two sheep.

The disappearance of ammonia from the rumen does not by itself show that ammonia enters the portal blood system of the ruminant animal; it may be completely metabolized by the rumen wall. A greater concentration of ammonia in ruminal venous blood as compared to arterial blood has, however, been measured (McDonald, 1948). Such a concentration difference demonstrates that ammonia that presumably has been absorbed from the rumen does enter the ruminal blood system and hence is carried to the liver. Lewis, Hill and Annison (1957) found a direct relationship between portal blood ammonia levels and rumen ammonia concentrations and estimated that up to 14 g of ammonia nitrogen could be absorbed from the rumen of a sheep daily.

Isotopic ammonia nitrogen placed in the rumen has also been found to enter the portal blood system (Kosharov et al., 1967). These workers found that the ^{15}N enrichment of the blood ammonia plus urea fraction in the portal system was greater than in the arterial system, thus absorption of ruminal ammonia must have occurred. Also the combined ammonia and urea fraction of the portal blood had the highest ^{15}N enrichment of any of the blood nitrogenous compounds that were examined, suggesting that this fraction was involved in the transfer of ^{15}N from the rumen to the liver.

Hogan (1961) and Bloomfield et al. (1963) studied factors affecting rumen ammonia absorption. The amount of ammonia absorbed was found to be dependent upon the concentration of ammonia in the rumen, thus the transfer of ammonia through the rumen wall into the portal blood system was said to occur by diffusion. Absorption was also found to be dependent upon pH; decreased absorption was observed at lower pH's and almost no absorption of ammonia from the rumen occurred at a pH of 4.5. Hogan (1961) related this pH effect to the equilibrium between free ammonia which can diffuse, and the ammonium ion which does not readily diffuse across membranes. Free, diffusable ammonia would be present in very low concentrations at a low rumen pH.

These observations lead to the conclusion that ammonia is lost from the rumen by absorption through the rumen wall and that at least some of this ammonia passes into the portal blood system and is carried to the liver of the animal.

B. Metabolism of ammonia in the animal

Lewis, Hill and Annison (1957) found that peripheral blood had a very low concentration of ammonia even when the concentration of ammonia

was quite high in portal blood, which indicates that the liver metabolizes most of the ammonia received from the digestive tract. It is commonly assumed that the majority of ammonia metabolized by the liver is converted to urea (Fontenot, 1971). There is considerable evidence, however, that absorbed rumen ammonia may be converted to compounds other than urea in the animal body.

In Section I of this study, it was pointed out that there was an apparent delay in the excretion of ^{15}N in the nitrogen of the urine of sheep when ^{15}N-ammonia was infused into the rumen, and that this suggested utilization of ammonia by the animal's body. Such utilization of ammonia may have occurred in the liver.

Land and Virtanen (1959) collected milk from cows fed isotopically labelled ammonia and found ^{15}N in the nitrogen of the milk 1 hour after feeding; after 14 hours the amide nitrogen in milk was the most highly enriched fraction. The amount of ^{15}N in the amide form was, however, only 15% of the total ^{15}N incorporated into the milk. Because of the relatively short time that was required for the milk to become labelled, and because the non-essential amino acids were more highly labelled than the essential amino acids in this experiment, it would appear that ammonia was converted to compounds other than urea in the animal's body, and that this conversion was not carried out by microorganisms in the rumen.

Faust et al. (1966) also speculated that substantial amounts of absorbed ammonia could have been converted into compounds other than urea in cows, particularly into amides. These authors used this possibility to explain the rapid appearance of ^{15}N in the amide fraction of the milk observed after feeding ^{15}N-urea to a cow. Abe and Kandatsu

(1969) measured a rapid rise in the isotopic enrichment of the non-protein nitrogen fraction of milk when ^{15}N-urea was administered to cows, and they also suggested that this was a result of ammonia conversions within the tissues of the animal.

Enzymic studies with ruminant livers also have shown that there is a possibility of the incorporation of ammonia into amino acids in the tissues of animals. Chalupa et al. (1970b) found that glutamate dehydrogenase activities in livers of sheep were higher in animals fed urea at a level of over 4% of the diet as compared to a control group of animals fed isolated soy protein as a nitrogen source. This increased activity, and the increased activity of the liver transaminases measured in the animals fed urea, suggest that glutamate formation may be important in the metabolism of ammonia in the liver. The reported depletion of reduced pyridine nucleotides in the liver caused by ammonia (Kutunama, Okada and Nishii, 1966), also supports the concept of glutamate formation in the liver.

Chalupa et al. (1970b) also reported that the inclusion of high levels of urea in the diet led to a reduction in liver enzymic activities of carbamoyl phosphate synthetase (ATP:carbamate phosphotransferase, 2.7.2.5), ornithine carbamoyltransferase (2.1.3.3) and arginase (3.5.3.1) which are three of the enzymes involved in urea synthesis in the liver. This again would suggest the possibility of the conversion of ammonia to compounds other than urea by the liver.

Experiments with non-ruminant animals have indicated that tissues of mammals can utilize ammonia. Foster, Schoenheimer and Rittenberg (1949) observed that ^{15}N-ammonia nitrogen was retained within the bodies of rats and humans when it was fed orally. Such a retention of labelled

ammonia by these non-ruminant species may well have been a result of liver ammonia metabolism.

Duda and Handler (1958) found that 30 minutes after ^{15}N-ammonia was given intravenously to rats 80% of the ammonia metabolized in the rat was located in the amide nitrogen, and that the enrichment of glutamine amide-nitrogen was seven times that of either urea, or glutamate, nitrogen at the respective maximums of enrichment. This experiment, although it suggests that ammonia was not directly metabolized to urea, must be considered with reservations, however, as ammonia presented to the peripheral system may be metabolized differently than that which enters portal system.

The synthesis of urea requires an input of energy of approximately 3.8 kcal of metabolizable energy per g of ammonia nitrogen converted to urea (Martin and Blaxter, 1965). Chalupa et al. (1970b) estimated that the energy cost of the formation of urinary urea in their sheep, which received over 4% of urea in their diet, would have been less than 1% of the digestible energy intake. Although this is not a large value, the synthesis of non-urea compounds from ammonia, which may not be recycled to the rumen to yield additional ammonia upon degradation would reduce the total flux through the urea pool and hence the energy cost of urea synthesis.

EXPERIMENTAL

A. Methods

^{15}N-ammonia and ^{14}C-urea were infused into the ruminal vein of sheep. Samples of urine were taken at various times over a 12 hour period and the ratio of ^{15}N to ^{14}C was determined and compared to this ratio in the infusate. Since it has been shown that very little ^{15}N-ammonia given orally reaches the kidneys and is excreted into the bladder as such (Sprinson and Rittenberg, 1949; Ulbrich and Scholz, 1966b), any decrease in the ratio of ^{15}N to ^{14}C in the urine with respect to the infusate would indicate a different retention of ammonia in the animal body as compared to urea.

B. Animal and feeding regimen

The animal used in this experiment was a Lincoln ewe weighing approximately 60 kg. A catheter was established in the right ruminal vein 6 days before the experiment was carried out (see Appendix E for the catheterization procedure). The catheter in this animal remained patent for 6 weeks.

The ewe was fed every 4 hours for 2 days before the experiment until the day of the experiment, during which it was fed a ration of 35 g of hay (2.3% nitrogen content on a dry matter basis) every hour.

C. Infusion and sampling procedures

A solution of ^{14}C-urea (Amersham-Searle Corporation, Alington Heights, Illinois) and ^{15}N-ammonium chloride (99 atoms % ^{15}N; Bio-Rad Laboratories, Richmond, California) in isotonic saline was infused into the right ruminal vein of a sheep at a rate of 0.152 ml/min for a period of 12 hours with the use of a variable speed infusion pump (series 950V,

Harvard Apparatus Co.). This rate resulted in the infusion of 1.79 μ moles of ^{15}N-ammonia and 21,700 dpm each minute.

Blood samples were taken from a jugular vein catheter at 1/2, 1, 2, 4, 6, 9, and 12 hours of the infusion period. Urine samples were collected from a bladder catheter up to the third hour of the experiment, at which time the ewe expelled the catheter. Subsequent urine samples were collected when the animal urinated.

D. Analytical methods

The ^{14}C content of urine and blood plasma was determined by counting 0.20 and 0.50 ml of each, respectively. Cab-O-Sil was added to the scintillation vials containing plasma samples to facilitate counting. Incubation of 6 of these samples with urease (EC 3.5.1.5), followed by the addition of trichloroacetic acid and solid CO_2 before counting demonstrated that there was insignificant activity in compounds other than urea or carbon dioxide. A similar procedure, without the use of urease showed that insignificant activity was present in the carbon dioxide of these samples.

All counting of radioactivity was done with Bray's (1960) scintillation fluid using a Nuclear Chicago Mark I Liquid Scintillation System (see Appendix G for the settings used on the Scintillation counter).

The specific activity of the blood and urinary urea was calculated from the dpm in the samples and from the concentration of urea, as determined by the method of Fawcett and Scott (1960), which is outlined in Appendix F. The Kjeldahl method of analysis was used to determine the nitrogen content of the urine. ^{15}N samples were analysed by the method used in Section I and given in Appendix D.

RESULTS

The metabolism of ammonia entering the portal system of the sheep can be examined by comparing the ratios of ^{15}N to ^{14}C in the infusate and in the urine. The average ^{15}N:^{14}C ratio in the urine was 0.077 μmoles of ^{15}N per dpm, or 93% of the ratio in the infusate (Table 8). This means that as far as urinary appearance was concerned, the nitrogen of ammonia and the carbon of urea when infused portally were nearly equivalent.

The rise in the specific activity of urea in the plasma and urine is illustrated in Figure 6. After 12 hours of infusion a plateau in specific activity was not reached. This is in contrast to results obtained by Cocimano and Leng (1967), who achieved steady state specific activity in blood urea after approximately 8 hours of intravenous infusion. Although steady state concentrations of isotope in the blood urea pool were not reached, it is possible to approximate the plateau of the specific activity of urea from Figure 6. From this value (50 dpm per umole of urea), and the measured infusion rate, an entry rate of 625 umoles of urea per day (17.5 g of nitrogen) was calculated for this sheep which received a total of 9.2 g nitrogen daily in the feed. This value agrees well with the entry rates measured by Ford and Milligan (1970).

Figure 6 also shows that the specific activity of urinary urea tended to be only slightly less than that of plasma urea at the same relative time, which would be expected since only a short length of time would be required for plasma urea to be filtered by the kidney and pass through the ureters into the bladder. The final value for the specific activity of the urinary urea was greater than that of plasma

Table 8. ^{15}N and ^{14}C concentrations and ratios in the infusate and urine of a sheep.

Sample	Sample time (hours)	Atoms % excess ^{15}N	$\frac{\mu moles}{^{15}N/ml}$	dpm/ml	$\frac{\mu moles\ ^{15}N}{dpm}$
Infusate	---	---	11.8	142,800	0.0826
Urine	3	0.0235	0.298	3,870	0.0770
Urine	9.7	0.0884	1.050	14,185	0.0740
Urine	12	0.1390	1.754	21,895	0.0801

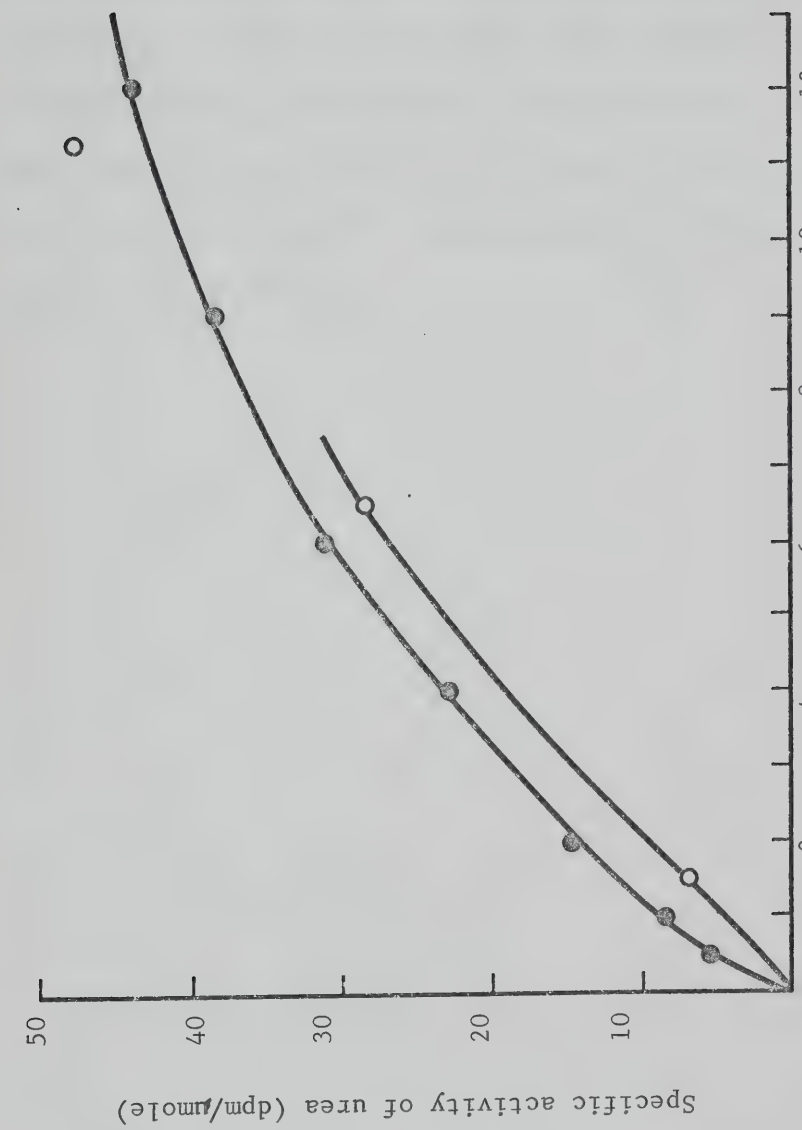

Figure 6. Plasma urea specific activity (●) and urinary urea specific activity (○) when ^{14}C-urea was infused into the portal blood system of a sheep. The specific activities of urine samples were plotted at points intermediate between the time the sample was obtained and the time the previous sample was obtained since the urine was produced over the total time interval.

urea. No experimental reason can be given for this physiologically illogical result.

In addition to the results reported above, glutamine amide-nitrogen, α-amino nitrogen, plasma urea nitrogen and blood plasma protein nitrogen were all isolated from the blood and analyzed for ^{15}N. Because of the small samples which could be isolated from the blood plasma, the sensitivity of detection of ^{15}N in these fractions was severely limited and no significant results were obtained for ^{15}N enrichment of these compounds in the plasma.

DISCUSSION

Sprinson and Rittenberg (1949) obtained results which showed that a negligible portion of ^{15}N-ammonia which enters the portal blood system of animals reaches the kidneys and is excreted into the bladder as such. In addition, Ulbrich and Scholz (1966b) presented data which show that most of the ^{15}N in the urine was in the form of urea when labelled ammonia was administered to the rumen. On the basis of these observations it can be concluded that the majority of the ^{15}N in the urine of this sheep was in the urea fraction. Thus, since the average ^{15}N:^{14}C ratio in the urine was 93% of the ratio in the infusate (Table 8), it can be concluded that approximately 93% of portally infused ammonia was metabolized by the liver to urea.

This result, derived with a sheep fed a high protein diet (2.3% nitrogen), can be compared with the results obtained by Sprinson and Rittenberg (1949), who found that, when ^{15}N-ammonium citrate was given orally to rats fed a high protein diet, from 78 to 96% of the isotope was recovered in the urine within 1 day, and that this isotope was predominantly in the urea fraction of the urine. This indicates that ammonia was converted primarily to urea in the bodies of the rats at a high level of protein intake. This agrees with the result obtained with the sheep in this experiment. The same workers, however, found that only approximately 30% of orally administered ^{15}N-ammonia was recovered in the urine after 1 day in rats fed a low protein diet. Even after 2 days only a maximum of 39% of the administered ammonia nitrogen was recovered in the urine when a low protein diet was consumed.

The results obtained by Sprinson and Rittenberg (1949) suggest that the percentage of absorbed ammonia which is converted to urea in the

liver may change depending upon the nitrogen intake of the animal. The possibility that the quantitative aspects of the conversion of ammonia to urea are dependent upon the diet was not studied in this experiment.

Although no evidence of any major conversion of ammonia to compounds other than urea by the liver was obtained in this experiment, the isotopic work of Land and Virtanen (1959), Abe and Kandatsu (1969), Faust et al. (1966) and that reported in Section I, provides evidence that ammonia can be utilized by the tissues of the sheep, even when high levels of nitrogen are fed. Although it did not appear that ammonia was extensively converted to compounds other than urea in the liver, such a conversion might occur in the rumen wall. The existence of enzymes of ammonia metabolism in the rumen wall has been demonstrated by various workers (Hoshino, Sarumaru and Morimoto, 1966; Chalupa et al., 1970a). The metabolism of the rumen wall will be examined in Section IV of this thesis.

SUMMARY

By infusing a mixture of ^{15}N-ammonia and ^{14}C-urea into the portal blood system of a sheep, it was found that approximately 93% of the ammonia in the portal blood of sheep was metabolized to urea in the liver when the sheep was fed a high protein hay diet. It was concluded that metabolism of ammonia to compounds other than urea in the liver of sheep was not significant when a diet with a relatively high content of protein was fed.

SECTION IV

ABSORPTION FROM THE RUMEN AND RUMEN WALL METABOLISM

INTRODUCTION

In Section I of this thesis results were obtained which suggested that amino acids were absorbed from the rumen. Amino acid absorption from the rumen has been previously demonstrated (Cook, Brown and Davis, 1965; Liebholz, 1971a), however, only very tentative estimates of the quantitative importance of this process are available. Amino acids, or some similar nitrogenous compounds, may also be transferred from the blood to the rumen (Liebholz, 1971a). This possibility was suggested to explain the source of nitrogen which enters the rumen of ruminants fed low protein diets (Section I and II).

The rumen wall, as well as being permeable to amino acids, may also metabolize ammonia to form amino acids (Hoshino, Sarumaru and Morimoto, 1966; Chalupa et al., 1970a). The relatively slow appearance of ^{15}N-ammonia nitrogen in the urine of sheep (Section I) and the rapid incorporation of ammonia nitrogen into amino acid and amide nitrogen in ruminants (Faust et al., 1966; Abe and Kandatsu, 1969) may also be explained by rumen wall metabolism, since little ammonia was incorporated into compounds other than urea by the liver in an experiment reported in Section III.

The experiments described in Section IV of this thesis were designed to determine the extent of amino acid absorption from the rumen. In addition, an attempt was made to measure the metabolic activity of rumen wall tissue and to determine if ammonia was metabolized to any significant extent by this tissue.

LITERATURE REVIEW

A. The absorption of amino acids from the rumen

Loss of amino acids from a washed rumen or rumen pouch is one technique which has been used to indicate amino acid absorption. Tsuda (1956), using this technique, demonstrated a decline in the concentration of glycine in a rumen pouch of a goat within 4 hours of administering a solution of amino acids. Leibholz (1971a) also observed that, when amino acids were present in the rumen in physiological concentrations, from 20 to 40% of the histidine and ammonia present in the rumen disappeared in a 4 hour period through either absorption or metabolism in the rumen wall. This worker concluded that up to 6% of the total nitrogen absorbed from the rumen could be in the form of α-amino nitrogen.

Candlish et al. (1970) carried out experiments with ^{14}C-labelled amino acids to demonstrate the absorption of amino acids from the rumen. They observed, in a rumen tied off at the omasum, that 68% of the ^{14}C-tryptophan initially present was lost from the rumen within 3 hours. Labelled tryptophan appeared in the portal blood of the sheep and in addition degradation products appeared in the urine, indicating that absorption was taking place.

Analysis of ruminal vein blood has yielded conflicting results concerning the absorption of amino acids from the rumen. Results which suggest amino acid absorption were obtained by Demaux et al. (1961), who found a 44% increase in α-amino nitrogen in rumen vein blood within 2 hours of administering a solution of amino acids into the rumen. Similarly, Cook, Brown and Davis (1965) also observed increases in ruminal vein blood amino acid concentrations when amino acids were placed

in a perfused rumen. In contrast to these results, which were obtained when relatively high concentrations of amino acids were present in the rumen, Smith (1959a) could not demonstrate any arteriovenous concentration difference in amino acids across the rumen when amino acids were present in the rumen in physiological concentrations.

The transfer of amino acids across rumen wall tissue has been shown in vitro (Leibholz, 1971b). Leibholz (1971b) provided evidence that the transport of amino acids across the rumen wall is an active, energy requiring process. This transfer was found to be specific for (l)-amino acids, was directionally specific and was inhibited by the metabolic inhibitors 2,4-dinitrophenol and iodoacetate. These findings, coupled with the observation that the transport of specific amino acids across rumen tissue is subject to competition from other amino acids (Cook, Brown and Davis, 1965; Leibholz, 1971b) conclusively show the existence of an active amino acid transporting system in the rumen wall.

The quantity of amino acid nitrogen absorbed from the rumen has only been roughly estimated. This is undoubtedly because the rapid degradation of amino acids in the functional rumen and small arteriovenous differences in amino acid concentrations made any measurement of absorption extremely difficult. The absorption of amino acids from the rumen, however, would be of such importance that considerable effort should be expended to determine its quantitative significance.

B. Metabolism in the rumen wall

a. General characteristics

An important aspect of the characterization of the metabolism of any tissue is the determination and measurement of the enzymes present in the tissue. Although only a limited amount of research has been done on

the enzyme complement of rumen mucosa (Young, Thorpe and DeLumen, 1969), a number of enzymes and enzyme systems have been identified. Enzymes of the tricarboxylic acid cycle are present in rumen mucosa as well as enzymes of the glycolytic pathway since Seto et al. (1970a, b) have demonstrated that these pathways are operational in ruminal tissue. In addition to the enzymes of these main pathways of metabolism, enzymes involved in gluconeogenesis and in the phosphogluconate oxidative pathway have been measured (Young, Thorpe and Delumen, 1969). Ruminal epithelial tissue also has the ability to metabolize volatile fatty acids (VFA) to form ketone bodies (Bush and Milligan, 1971), and various enzymes involved in nitrogen metabolism have been demonstrated in this tissue (Hoshino, Sarumaru and Morimoto, 1966; Ide, 1969).

It is apparent from the enzyme compliment present in rumen mucosa that this tissue has the potential for carrying out numerous metabolic reactions. A good estimate of the amount of metabolic activity normally taking place in the tissue, however, can only be obtained from a measurement of the oxygen utilization of the tissue. No in vivo measurement of oxygen consumption of rumen tissue has been reported to date, however, numerous results from in vitro measurements are present in the literature. Results obtained by Hird and Weidemann (1964) show that up to 10 ml of oxygen were consumed/hr/g of dry rumen epithelium when substrate was present. This was essentially the same as the oxygen uptake of liver slices, which was measured at a maximum of 12 ml/hr/g of dry tissue when butyrate was present as the substrate. This comparable rate of oxygen utilization by the liver and ruminal epithelial tissue in vitro may indicate comparable metabolic rates in vivo (Holliday et al., 1967), and thus would suggest that the metabolic activity of ruminal

tissue is high relative to the tissues of the rest of the animal.

b. Metabolism of nitrogenous compounds

Considerable attention has been directed towards studying the possibilities of the metabolism of nitrogenous compounds in the rumen wall. As a result NAD- and NADP-linked glutamate dehydrogenases which may play an important role in ammonia fixation, have been demonstrated in rumen mucosal tissue (Hoshino, Sarumaru and Morimoto, 1966; Chalupa et al., 1970a). The activity of glutamate dehydrogenase in this tissue, however, is only approximately 20% that in the liver (Chalupa et al., 1970a, b), thus ammonia fixation by the production of glutamate may not be of great significance in rumen mucosa.

Other enzymes of ammonia assimilation and nitrogen metabolism have been found in the rumen wall. Glutamine synthetase is present in small amounts in rumen mucosa (Hoshino, Sarumaru and Morimoto, 1966). Ide (1969) has reported that some enzymic activity was found for ornithine carbamoyltransferase, which is an enzyme of urea production. No carbamoyl phosphate synthetase activity was however, found. Transaminases are also present in rumen epithelium (Chalupa et al., 1970a).

There are few reports in the literature concerning the extent of metabolism of nitrogenous compounds in the rumen wall tissue as determined by in vitro techniques. Kurilov and Kosarov (1969) claim to have demonstrated the synthesis of amino acids from ammonia using rumen tissue. Pyruvate had a stimulatory effect on this synthesis.

One of the few in vivo experiments which directly shows the metabolism of a nitrogenous compound by the rumen wall was reported by Aliev and Kosarov (1967). They observed that the blood draining a rumen pouch contained labelled urea when $^{15}NH_4Cl$ was added to the pouch. The authors

concluded that urea was formed in the rumen wall. This conclusion, would appear to be contradictory to the observation that enzymes of urea synthesis were virtually absent from rumen mucosal tissue (Ide, 1969).

These observations on rumen wall nitrogen metabolism, even though they are not conclusive, show the possibility of the metabolism of nitrogen compounds in the rumen wall. No indication, however, is given concerning the extent or importance of any such metabolism.

c. Metabolism of fatty acids

The metabolism of VFA in the rumen wall was initially postulated to account for the fact that the blood draining the rumen contained less butyrate, relative to acetate and propionate, than would be expected on the basis of the VFA concentrations in the rumen (Kiddle, Marshall and Phillipson, 1951; Masson and Phillipson, 1951). It has since been found that butyrate is metabolized by the rumen wall tissue to a greater extent than any other VFA (Pennington, 1954), and that 3-hydroxybutyrate is the main end product of butyrate metabolism *in vivo* (Bergman, 1971).

The amount of butyrate metabolized by the rumen wall has been estimated by various workers. Bergman and Wolff (1971), by combining estimates of rumen production with measurements of net absorption from the rumen have concluded that up to 90% of absorbed butyrate does not reach the portal blood system. This measurement is in agreement with the estimates made by Stevens (1970), that up to 85% of the butyrate which crosses the rumen wall is metabolized in the tissue.

Propionate has been suggested to be metabolized in rumen mucosal tissue, with lactate being an end product of metabolism (Pennington and Sutherland, 1956; Taylor and Ramsey, 1965). Pennington and Sutherland

(1956) have proposed that the pathway of this conversion of propionate to lactate involves carboxylation and succinate formation. Stevens (1970), from in vitro experiments, has estimated that up to 65% of the propionate transferred across the rumen wall is metabolized. Bergman and Wolff (1971), from in vivo measurements, found that up to 50% of the propionate absorbed from the rumen did not reach the portal blood system and similarly concluded that a large portion of absorbed propionate is metabolized in the rumen wall. In contrast to these results, however, Weigand, Young and McGilliard (1972) found that only 2.3% of absorbed propionate was converted to lactate in calves. More research is thus necessary in this area.

Ketone bodies are also formed in the rumen wall from acetate (Pennington, 1954). The extent of metabolism of acetate by rumen tissue is not as great as that of propionate or butyrate, however, Stevens (1970) and Bergman and Wolff (1971) have reported that 45 and 30% respectively of the acetate absorbed from the rumen is metabolized in the rumen wall.

In addition to the short-chain fatty acids, branched-chain fatty acids and long-chain fatty acids are extensively metabolized by rumen epithelium (Annison and Pennington, 1954; Hird, Jackson and Weidemann, 1966). Ruminal tissue then, has the capacity to metabolize the fatty acids to which it is exposed. Since there is extensive passage of fatty acids through this tissue, significant metabolism of these substrates likely occurs in the rumen wall.

EXPERIMENTAL

A. Methods

Blood samples were taken simultaneously from catheters in the carotid artery and right ruminal vein of sheep when the sheep were in a steady metabolic state. The arteriovenous concentration differences were determined for ammonia, urea, glutamate, glycine, acetate, propionate, 3-hydroxybutyrate, carbon dioxide and oxygen after rumen vein blood concentrations were corrected to the same water content as carotid blood on the basis of hemoglobin concentrations.

In an attempt to quantitate the net daily absorption or utilization of these substances, acetate-1-^{14}C (Amersham-Searle; Arlington Heights, Illinois) and polyethylene glycol (PEG) were infused into the rumen of the sheep at a rate of approximately 300,000 dpm/min and 6 mg/min, respectively, for a period of 2 days. The amount of radioactivity in the rumen fluid was related to the concentration of PEG in the rumen fluid and the rate of disappearance of the PEG and thus the total amount of radioactivity passing into the omasum daily could be determined. The difference between the total radioactivity infused into the rumen and that entering the omasum was assumed to be equal to the amount absorbed. The rumen arteriovenous concentration differences of the various metabolites could then be compared to that of the isotope to determine the total quantities of metabolites absorbed into the ruminal blood system or utilized by the rumen.

An effort was also made to determine if amino acids were formed by rumen wall tissue by incubating rumen papilli with various substrates including ammonia, glutamine, 2-oxoglutarate, glyoxalate, pyruvate, oxaloacetate, glucose and VFA in various combinations.

B. Animals and feeding regimen

The three animals used in these experiments were all Rambouillet wethers, weighing approximately 40 kg. Before an experiment was started, a carotid loop and a rumen fistula were established in each animal. A catheter was also inserted into the right ruminal vein (Appendix E), either in the initial operation, or 1 week later. Experiments were not carried out with the sheep until at least 10 days had been allowed for recovery from surgery.

The sheep were kept in metabolism cages throughout the experimental period and were fed during, and at least 1 week before the experiment, from a mechanical feeder which dropped feed for the animals every 10 minutes (Ford, 1969). The diets which were used in the experiments were alfalfa-grass hays (hay 1, hay 2), straw and barley, which contained 2.2, 1.9, 1.5 and 1.7% nitrogen on a dry matter basis, respectively. In addition, in one experiment NH_4Cl was continuously infused at the rate of 3.22 g N/day into the rumen of a sheep fed hay 1.

C. Sampling procedures

^{14}C-acetate and PEG were infused into the rumen of the sheep for a period of 1 day before any samples were taken so that the concentrations of these two substances would be relatively stable in the rumen during the sampling period. Every 3 hours samples were taken from the rumen of the sheep with a plastic tube fitted to a 50 ml syringe. The pH of each sample was measured and the samples were frozen until analysis could be carried out.

Every 3 hours 5 ml of blood were simultaneously withdrawn from the carotid and rumen vein catheters into heparinized plastic

syringes. Blood pH, pO_2 and pCO_2 were determined on each blood sample immediately after the samples were obtained. Two drops of heparin were then added and the samples were frozen and stored for analysis.

D. Oxygen consumption of sheep

The oxygen consumption of the sheep was determined from measurements of both the rate of flow of air (corrected to STP) through a face mask on the sheep and the oxygen concentration of inspired and expired air. Air flows were measured with a dry-gas meter and oxygen concentrations of air were measured with a Beckman F-3 oxygen analyzer[1].

E. In vitro incubation procedure

Rumen wall tissue, obtained from a local abbatoir, was placed on ice as soon as it was obtained and transported to the laboratory. Rumen papilli were cut off this tissue with scissors. Approximately 400 mg wet weight of these papillae were incubated with various substrates in 4 ml of an isotonic solution of Krebs' bicarbonate buffer (Umbreit, Burris and Stauffer, 1959) in rubber stoppered 25 ml Erlenmeyer flasks at 39 C. The metabolism of this tissue was terminated after 3 hours by the addition of 1 ml of 70% $HClO_4$ and the resulting solution was analyzed.

F. Analytical methods

a. Radioactivity measurements

Blood samples were prepared for scintillation counting by adding an equal volume of freshly prepared 10% (w/v) trichloroacetic acid to precipitate the protein. This mixture was left for 30 minutes in an ice

[1] Beckman Instruments, Inc., Fullerton, California, U.S.A.

bath then the samples were centrifuged at 10,000 x g for 10 minutes and 0.5 ml of the supernatant was added to a scintillation vial. Solid CO_2 was added to the sample in the scintillation vial to purge any radioactive CO_2 present. Fifteen ml of scintillation fluid (Bray, 1960) were then added to the vial and the samples were counted.

The total radioactivity of the rumen fluid was determined in samples prepared by adding 0.25 ml of strained rumen fluid to a scintillation vial; 0.25 ml of 10% (w/v) trichloroacetic acid was added to make the solution acidic and solid CO_2 was then added to purge any radioactive CO_2 present. Two ml of NCS Solubilizer were added to the sample and then 1 ml of a benzoyl peroxide solution (20%, w/v, in toluene) was added. The mixture was allowed to stand for 48 hours to solubilize the organic constituents of the rumen fluid and to allow decolorization to proceed. After this period, the material was heated for 2 hours at 50 C, cooled and 12 ml of scintillation fluid (Bray, 1960) were added. With this method of sample preparation counting efficiencies of 45% were achieved and there was excellent agreement between duplicates.

All radioactivity measurements were made with the Nuclear Chicago Mark I Liquid Scintillation System employing the settings described in Appendix G.

b. pH and gas content of blood

Whole blood was held in a closed syringe in ice until the pH, pCO_2 and pO_2 could be determined. Analyses were always complete within 1/2 hour after the blood was withdrawn from the animal. The Radiometer Blood Micro System (BMS3)[1] was used for the blood analysis with

[1] Radiometer, Copenhagen, Denmark.

the electrodes for pH($G_2$98A), PO_2(E5 047) and pCO_2(E5 036) mounted in a bath maintained at 39 C.

Hemoglobin was determined as cyanomethemoglobin (Hycell Inc., Appendix F) and the percentage saturation of the hemoglobin was determined with the Blood Gas Calculator (type BGC1)[1]. The oxygen content of the blood was calculated assuming that 1.34 ml of oxygen combines with 1 g of hemoglobin at 100% saturation (Foex et al., 1970).

The carbon dioxide content of the blood was determined with the Blood Gas Calculator (type BGC1)[1].

c. Deproteinization of blood

In all chemical determinations, except the determination of hemoglobin, blood was deproteinized by the addition of an equal volume of 10% (w/v) perchloric acid. This solution was allowed to stand in an ice bath for 10 minutes and then was centrifuged at 10,000 x g. The supernatant from this centrifugation was neutralized with 5N KOH, made up to a known volume in a graduated conical glass centrifuge tube, put in an ice bath for 10 minutes, then centrifuged at 10,000 x g for 10 minutes. The supernatant from this centrifugation was then saved for analysis.

d. Volatile fatty acid analysis

Two ml of $HClO_4$ deproteinized blood were used for VFA analysis. The neutralized samples were made basic by the addition of 0.1 ml of 1N KOH and lyophilized in conical centrifuge tubes. When the material was completely dry the residue was taken up in 0.2 ml of 12.5% (w/v) metaphosphoric acid and 2 µl of this solution were injected into a gas chroma-

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[1] Radiometer, Copenhagen, Denmark.

tograph for analysis.

Rumen fluid was prepared for VFA analysis by adding 1 ml of 25% (w/v) meta-H_3PO_4 to 5 ml of rumen fluid.

An Aerograph Gas Ghromatograph with a flame ionization detector (Model 600-D) was used to separate the volatile fatty acids. The column in this chromatograph (0.32 cm x 122 cm) was packed with Porapak Q, treated with 2% H_3PO_4 (w/w) in a 70% aqueous ethanol solution (Kellogg, 1969). Column and injector temperatures were 235 and 250 C, respectively. Nitrogen was used as the carrier gas and a flow rate of 20 ml/min was maintained.

Concentrations of VFA in the samples were determined by comparing peak areas, measured by triangulation, with peak areas obtained from standard solutions.

e. Chemical determinations

A detailed description of each of the following chemical procedures is given in Appendix F.

PEG was determined by the method of Smith (1959b). Rumen ammonia concentrations were determined by the method of Fawcett and Scott (1960) using the deproteinized rumen fluid obtained in the determination of PEG.

Blood that was deproteinized with $HClO_4$ was used for the determination of ammonia (Fawcett and Scott, 1960). Glutamate (Bernt and Bergmeyer, 1965) and 3-hydroxybutyrate (Williamson and Mellanby, 1965) were determined enzymatically. Glycine was analysed by the method of Alexander, Landwehr and Seligman (1945) as modified by Christenson, Riggs and Ray (1951).

H. Statistical methods

The paired 't' test (Steel and Torrie, 1960) was used to determine if the rumen arteriovenous concentration differences of various metabolites were statistically significant.

RESULTS

The daily dry matter, nitrogen and energy intakes of the sheep in the five experiments are summarized in Table 9. The VFA and ammonia concentrations and the pH of the rumen contents of these sheep are given in Table 10.

A. Quantitative estimates of absorption into ruminal blood

a. Determination with acetate-1-^{14}C

The results obtained in an attempt to measure the amounts of metabolites absorbed into the ruminal blood stream by comparing their arteriovenous concentration differences across the rumen to that of intraruminally infused isotope did not appear logical. As a result, an effort was made to measure the quantity of acetate absorbed from the rumen daily so that a quantitative estimate of absorption of other metabolites could be made from their arteriovenous concentration differences relative to that of acetate. In the process of determining the entry rate of acetate into the ruminal pool it was found, by steam distillation, that only approximately 2% of the isotope in samples of rumen contents was volatile and was thus recovered in the distillate in the distillation process, although 85 to 95% of the unlabelled ruminal VFA were recovered. The same results were obtained when infusate containing ^{14}C was steam distilled with a known quantity of unlabelled acetate. When, however, known acetate-2-^{14}C was steam distilled from a solution containing unlabelled acetate, the isotope and unlabelled acetate were distilled over at the same rate and recovery of each was 95%.

From these results the following possibilities were indicated: the

Table 9. Intake, output and apparent digestibility of dry matter, energy and nitrogen and nitrogen retention of sheep.
(Mean values with their standard errors* for total daily collections from each sheep)

Diet	Sheep Number	Dry Matter			Gross Energy			Nitrogen				
		Intake (g/day)	Digested (g/day)	Digestibility (%)	Intake (kcal/day)	Digested (kcal/day)	Digestibility (%)	Intake (g/day)	Digested (g/day)	Digestibility (%)	Urine (g/day)	Retained (g/day)
Hay 1	9238	420	260±13	61.9±3.1	1865	1133±58	60.7±3.1	9.28	6.46±0.25	69.6±2.8	9.27±0.79	-2.81±0.93
Hay 2	9244	428	266±4	62.2±0.9	1866	1136±20	60.9±1.1	8.22	5.56±0.06	67.6±0.8	7.33±0.57	-1.77±0.59
Hay & Ammonia	9242	420	271±14	64.6±3.3	1865	1194±64	64.0±3.4	12.50	9.94±0.21	79.5±1.7	10.82±0.46	-0.88±0.57
Straw	9238	442	219±7	49.5±1.6	1962	958±39	48.8±2.0	6.67	3.36±0.11	50.3±1.7	6.38±0.79	-3.02±0.72
Barley	9244	346	313±4	90.6±1.2	1540	1394±17	90.6±1.1	5.81	5.28±0.06	90.9±1.2	7.15±0.62	-1.90±0.69

* Number of observations for hay 1, hay 2, hay-ammonia, straw and barley diets were 4, 4, 4, 4 and 3 respectively.

Table 10. Ruminal pH and volatile fatty acid and ammonia concentrations.
(Mean values with their standard errors*)

Diet	pH	Ammonia (µmoles/ml)	Volatile fatty acids			
			Acetate (µmoles/ml)	Propionate (µmoles/ml)	Butyrate (µmoles/ml)	Total VFA** (µmoles/ml)
Hay 1	6.5	7.6±0.9	46.7±2.9	16.0±1.4	4.2±0.3	66.9±4.4
Hay 2	6.6	11.5±0.6	45.5±1.3	10.8±0.4	4.5±0.3	60.8±1.5
Hay & ammonia	6.5	14.1±2.9	37.4±2.9	13.6±1.4	3.9±0.2	56.8±5.4
Straw	6.6	6.1±0.3	37.5±1.7	10.5±0.5	4.6±0.4	52.6±2.3
Barley	6.0	12.5±0.3	30.0±1.9	6.8±0.4	7.0±0.4	44.4±2.2

* Number of samples analysed were 6 for ammonia determinations and 5 for volatile fatty acid determinations.
** Acetate + propionate + butyrate

isotope supplied by Amersham-Searle Corp. was not acetate-1-^{14}C, there was microbial assimilation or metabolism of the isotope before infusion or a reaction had occurred between the ^{14}C-acetate and PEG in the infusate solution before infusion. In order to test the latter possibility a solution of PEG and acetate-2-^{14}C was prepared and a portion of this solution was steam distilled daily for 3 days. ^{14}C-labelled acetate distilled at the same rate as unlabelled acetate from this solution. On this basis it was concluded that the isotope supplied by Amersham-Searle Corp. was not acetate-1-^{14}C or that microbial assimilation of the isotope in the infusate had taken place. No further attempt has been made to identify the form of the isotope in the infusate.

b. Determination by rumen arteriovenous acetate concentration differences

Since a quantitative estimate of the absorption of metabolites from the rumen into ruminal blood could not be obtained by reference to a ^{14}C-acetate tracer, estimates were based on the rumen arteriovenous acetate concentration differences and the quantity of acetate calculated to be absorbed from the rumen daily. Absorbed acetate, in this case, was assumed to be the difference between the daily production of acetate in the rumen and the amount of acetate passing into the omasum daily. The measured amount of acetate flowing into the omasum was calculated from the following equation:

$$\text{acetate entering omasum (mmoles/day)} = \text{acetate concentration (mmoles/ml of rumen fluid)} \times \frac{\text{g PEG infused/day}}{\text{g PEG/ml of rumen fluid}}$$

The equations used to estimate the effective production rates of VFA in the rumen were those of Leng, Corbett and Brett (1968) and are based on the measured rumen concentrations of VFA.

These equations and their standard errors are shown below:

acetate production (mmoles/min) =

0.864 (0.048(rumen concentration, mmoles/l) - 0.648) $\pm$ 0.006

propionate production (mmoles/min) =

1.0 (0.037 (rumen concentration, mmoles/l) - 0.078) $\pm$ 0.005

butyrate production (mmoles/min) =

0.618 (0.032 (rumen concentration, mmoles/l) + 0.085) $\pm$ 0.004

Table 11 gives a summary of the estimated VFA production rates in the rumen based on these equations. The quantities of total VFA flowing into the omasum daily were also calculated by reference to the concentration of PEG in the rumen fluid and the infusion rate of PEG. The differences between these values were the quantities of VFA absorbed from the rumen daily (Table 11).

Values for blood flow past the rumen (Table 12) are based on the estimated daily absorption of acetate and the arteriovenous difference in acetate concentration. Blood flow estimates obtained in this manner are only approximate since the right ruminal vein blood may not be representative of the blood flowing past the whole rumen (Barcroft, McAnally and Phillipson, 1944). The values are useful, however, in estimating absorption and for comparing with results of other workers. The blood flow estimates obtained in this manner ranged from 10 to 53 ml/min/kg body weight in the sheep.

B. Net absorption of nitrogenous compounds into ruminal blood

The carotid concentrations of urea, glutamate and glycine are shown in Table 13. No values are shown for carotid ammonia concentrations since whole blood was stored for analysis and thus the concentration of

Table 11. Measurement of the quantity of rumen fluid entering the omasum daily and estimates of daily ruminal volatile fatty acid production and absorption.

(Mean values; their standard errors given where appropriate; numbers of observations in parentheses)

Diet	Estimated production rates of ruminal VFA			PEG Infused (g/day)	Rumen PEG Concentration (mg/ml)	Rumen fluid entering omasum (l/day)	Estimated absorption rates of ruminal VFA		
	Acetate (m/day)	Propionate (m/day)	Butyrate (m/day)				Acetate (m/day)	Propionate (m/day)	Butyrate (m/day)
Hay 1	1.99	0.74	0.20	11.10	1.77±0.11 (7)	6.27	1.70	0.64	0.17
Hay 2	1.92	0.46	0.20	9.22	1.86±0.06 (11)	4.96	1.69	0.41	0.18
Hay & ammonia	1.45	0.60	0.19	7.91	1.91±0.06 (8)	4.14	1.28	0.54	0.17
Straw	1.44	0.45	0.20	8.03	1.20±0.03 (11)	6.69	1.19	0.38	0.17
Barley	0.98	0.25	0.27	7.78	1.60±0.03 (10)	4.86	0.83	0.22	0.24

Table 12. Estimates of rumen blood flow.

(Mean values; their standard errors given where appropriate; numbers of observations in parenthesis)

Diet	Sheep Number	Sheep weight (kg)	Hemoglobin concentration (g/100 ml)	Estimated acetate absorption (moles/day)	(V-A) acetate concentration* (μmoles/ml)	Blood flow (ml/min)	Blood flow (ml/min/kg body weight)
Hay 1	9238	40	12.10±0.19 (11)	1.70	0.82±0.43 (4)	1440	36
Hay 2	9244	39	8.01±0.14 (11)	1.69	1.03±0.26 (5)	1140	29
Hay & ammonia	9242	42	9.49±0.16 (10)	1.28	2.13±0.25 (5)	420	10
Straw	9238	39	11.64±0.23 (7)	1.19	0.40±0.16 (4)	2070	53
Barley	9244	40	9.21±0.13 (10)	0.83	0.41±0.13 (4)	1410	35

* Right ruminal vein blood acetate concentration (corrected to the same water content as carotid blood on the basis of hemoglobin concentration) - carotid artery acetate concentration.

Table 13. Carotid concentrations, arteriovenous concentration differences and net daily absorption of nitrogenous compounds into ruminal blood.

(Mean values; their standard errors given where appropriate; numbers of observations in parenthesis)

Diet	Carotid concentration			Venous-arterial concentration differences+				Net absorption into rumen blood≠		
	Urea (μmoles/ml)	Glutamate (μmoles/ml)	Glycine (μmoles/ml)	Ammonia (μmoles/ml)	Urea (μmoles/ml)	Glutamate (μmoles/ml)	Glycine (μmoles/ml)	Ammonia (moles/day)	Glutamate (moles/day)	Glycine (moles/day)
Hay 1	2.99±0.13 (10)	0.350±0.014 (10)	0.724±0.030 (9)	0.320±0.121** (10)	-0.029±0.164 (10)	0.077±0.028** (10)	0.145±0.050** (9)	0.664**	0.160**	0.301**
Hay 2	4.02±0.29 (10)	0.164±0.003 (10)	0.713±0.016 (10)	0.229±0.038*** (10)	-0.293±0.236 (10)	0.011±0.001** (10)	-0.002±0.022 (10)	0.376***	0.018**	-0.003
Hay & Ammonia	4.27±0.19 (9)	0.385±0.011 (9)	0.753±0.036 (7)	1.17 ±0.076*** (9)	-0.318±0.318 (9)	0.026±0.017 (9)	-0.040±0.047 (9)	0.708***	0.016	-0.024
Straw	2.83±0.10 (9)	0.323±0.006 (9)	1.004±0.023 (10)	0.156±0.034*** (7)	-0.002±0.144 (6)	0.025±0.003*** (6)	0.147±0.038*** (7)	0.465***	0.074***	0.438***
Barley	3.35±0.25 (7)	0.169±0.022 (8)	0.756±0.100 (8)	0.166±0.119 (7)	-0.034±0.237 (7)	0.004±0.008 (8)	-0.020±0.037 (8)	0.337	0.008	-0.041

+ Rumen vein blood is corrected to the same water content as carotid blood on the basis of hemoglobin concentration.
≠ Calculated from arteriovenous concentration differences relative to acetate and the assumption that no metabolism of the absorbed acetate occurred in the rumen wall.
* Significant at $P < .10$.
** Significant at $P < .05$.
***Significant at $P < .01$.

ammonia in the samples spontaneously increased with time. This change in blood ammonia has been previously reported (Preston, 1969). A significant positive correlation was observed between rumen ammonia concentration and carotid blood urea concentration ($r = 0.87$) in agreement with other results (Lewis, 1957). No significant correlations were observed between either carotid glycine, or glutamate, concentrations and rumen ammonia concentrations.

A significant difference was observed between ruminal arterial and venous blood ammonia concentration in all cases, except in the sheep fed barley (Table 13). In addition, glutamate was present in higher concentrations in rumen vein blood than arterial blood in all cases, but the difference was not statistically significant for the hay + ammonia and barley treatments. In the sheep fed the hay 1 and straw diets glycine was also present in higher concentrations in the rumen blood than the arterial blood (Table 13). This would suggest that the net absorption of at least some amino acids from the rumen into ruminal blood may occur.

The net absorption of ammonia, glycine and glutamate was calculated on the basis of their rumen arteriovenous differences relative to acetate, which was assumed to pass through the rumen wall without being metabolized. From 4.8 to 9.9 g of ammonia nitrogen were absorbed into the ruminal blood of the sheep daily (Table 13). Up to 6.1 g glycine nitrogen and 2.2 g glutamate nitrogen were absorbed into ruminal blood daily in the sheep where absorption was significant (Table 13). No estimates of the extent of urea recycling to the rumen were made for these sheep since no significant arteriovenous differences in urea concentration were observed across the rumen (Table 13).

C. Absorption and metabolism of ruminal volatile fatty acids

The carotid concentrations of acetate, propionate and 3-hydroxybutyrate are given in Table 14. No butyrate could be detected in arterial blood. No relationship was observed between rumen acetate, propionate and butyrate concentrations and carotid concentrations.

The VFA and 3-hydroxybutyrate concentrations of ruminal vein blood were significantly higher than those of arterial blood at the 10% level of significance in all cases except that of the sheep fed the hay 1 diet. A high variability in the estimates of rumen arteriovenous concentration differences was obtained because of the difficulties inherent in simultaneous arterial and venous sampling. In addition numbers of samples that could be analyzed were rather low.

The net absorption of propionate and 3-hydroxybutyrate into rumen venous blood was calculated on the basis of their rumen arteriovenous differences relative to acetate. In all cases the net absorption of acetate into ruminal blood was assumed to be 100% of the amount estimated to be available for absorption (Table 11). Thus, 71 to 134% of the estimated amount of propionate available for absorption from the rumen was calculated to appear in the ruminal vein blood if all of the acetate available for absorption appeared in the venous blood (Tables 11, 14). The main product of butyrate metabolism in the rumen wall, 3-hydroxybutyrate (Bergman, 1971), was calculated to account for 64 to 200% of the butyrate apparently absorbed from the rumen (Tables 11, 14).

D. Blood gases and blood pH

The partial pressure of blood oxygen was higher in the arterial blood than in the right rumen vein blood in all experiments (Table 15).

Table 14. Carotid concentration, arteriovenous concentration differences and net daily absorption of acetate, propionate and 3-hydroxybutyrate into ruminal blood.

(Mean values; their standard errors given where appropriate; numbers of observation in parenthesis)

Diet	Carotid concentration			Venous-arterial concentration differences+			Net absorption into rumen blood≠		
	Acetate (μmoles/ml)	Propionate (μmoles/ml)	BHB (μmoles/ml)	Acetate (μmoles/ml)	Propionate (μmoles/ml)	BHB (μmoles/ml)	Acetate (moles/day)	Propionate (moles/day)	BHB (moles/day)
Hay 1	2.63±0.12(4)	0.26±0.02(4)	0.231±0.018(10)	0.82±0.43(4)	0.22±0.05(4)	0.084±0.039(10)*	1.70	0.456	0.175*
Hay 2	3.89±0.05(5)	0.29±0.01(5)	0.325±0.011(10)	1.03±0.26(5)**	0.23±0.05(5)**	0.070±0.009(10)***	1.69**	0.378**	0.115***
Hay & Ammonia	2.86±0.08(5)	0.33±0.01(5)	0.271±0.019(9)	2.13±0.25(5)***	0.65±0.06(5)***	0.258±0.017(9)***	1.28***	0.393***	0.156***
Straw	2.62±0.18(4)	0.24±0.01(4)	0.387±0.017(9)	0.40±0.0I6(4)*	0.17±0.07(4)*	0.114±0.015(6)***	1.19*	0.507*	0.340***
Barley	2.56±0.11(4)	0.33±0.01(4)	0.406±0.016(8)	0.41±0.13(4)*	0.11±0.02(4)**	0.117±0.020(9)***	0.83*	0.223**	0.238***

+ Rumen vein blood is corrected to the same water content as carotid blood on the basis of hemoglobin concentration.
≠ Calculated from the arteriovenous concentration differences relative to acetate and the assumption that no metabolism of absorbed acetate occurred in the rumen wall.
* Significant at $P < .10$.
** Significant at $P < .05$.
***Significant at $P < .01$.

Table 15. Carotid and right rumen vein blood pH, pCO_2 and pO_2.
(Mean values with their standard errors; numbers of observations given in parenthesis)

Diet	Carotid blood			Right rumen vein blood		
	pH	pCO_2 (mm)	pO_2 (mm)	pH	pCO_2 (mm)	pO_2 (mm)
Hay 1	7.451±0.004 (9)	33.00±0.62 (9)	77.12±1.93 (8)	7.393±0.004 (9)	40.05±0.43 (9)	51.00±0.65 (8)
Hay 2	7.478±0.006 (10)	32.10±0.82 (10)	83.00±1.49 (9)	7.377±0.004 (10)	42.25±0.61 (10)	49.44±0.71 (9)
Hay & ammonia	7.427±0.004 (11)	33.35±0.91 (10)	78.50±1.32 (10)	7.229±0.005 (11)	54.10±0.92 (10)	53.00±1.55 (10)
Straw	7.445±0.004 (9)	32.38±0.94 (8)	81.67±1.21 (9)	7.370±0.005 (9)	40.33±0.47 (9)	52.22±0.64 (9)
Barley	7.522±0.015 (10)	32.55±1.00 (10)	79.45±1.33 (10)	7.425±0.008 (10)	42.75±1.10 (10)	51.45±1.03 (10)

In all animals, except the sheep fed the barley diet, there was more carbon dioxide in the venous blood than the arterial blood (Table 16), indicating the net input of carbon dioxide into ruminal blood. A statistically significant amount of carbon dioxide, however, was absorbed from the rumen only in the sheep fed the straw and hay 1 diets (Table 16).

Blood pH was lower in the venous blood than in the arterial blood (Table 15). This would be expected in terms of the greater carbon dioxide content of the venous blood and the greater VFA content of ruminal vein blood.

Table 16. Carotid concentrations and arteriovenous concentration differences of O_2 and CO_2 in rumen blood and estimates of sheep and ruminal gas utilization corrected to standard temperature and pressure.

(Mean values; their standard errors given where appropriate; numbers of observations in parenthesis)

Diet	Carotid concentration		V-A concentration difference+		CO_2 absorbed from rumen (moles/day)	O_2 utilization by the rumen^S			Oxygen consumption of sheep (ml/min)
	O_2 (μmoles/ml)	CO_2 (μmoles/ml)	O_2 (μmoles/ml)	CO_2 (μmoles/ml)		moles/day	ml/min	ml/min/kg rumen tissue≠	
Hay 1	6.69±0.12 (8)	23.35±0.75 (9)	-1.34±0.080*** (8)	3.22±0.99** (9)	6.48**	2.78***	43.2***	50***	-
Hay 2	4.61±0.30 (9)	24.32±0.59 (9)	-1.11±0.039*** (9)	1.00±0.62 (9)	1.64***	1.82	28.3***	32***	172
Hay & ammonia	5.26±0.14 (8)	22.30±0.54 (10)	-1.33±0.12*** (8)	0.63±0.63 (9)	0.381	0.804***	12.5***	14***	246
Straw	6.58±0.13 (6)	22.56±0.54 (6)	-1.27±0.06*** (6)	1.67±0.77* (6)	4.98*	3.78***	58.8***	70***	-
Barley	5.33±0.08 (9)	26.93±1.09 (9)	-1.07±0.04*** (9)	-0.10±1.16 (9)	-0.20	2.17***	33.8***	39***	142

\+ Venous-arterial concentration difference; rumen vein blood is corrected to the same water content as carotid blood on the basis of hemoglobin concentration.

≠ Based on the assumption that rumen tissue is 2.15% of live body weight (Ingle, Bauman & Garrigus, 1972).

* Significant at $P < .10$.

** Significant at $P < .05$.

*** Significant at $P < .01$.

S Calculated from the arteriovenous concentration difference relative to acetate and the assumption that no metabolism of absorbed acetate occurred in the rumen wall.

DISCUSSION

A. Estimates of absorption into ruminal blood

a. Validity of the estimates

The flow of blood past the rumen is difficult to measure because two large veins drain the rumen. In addition estimating absorption of metabolites into ruminal blood is made more difficult by the possibility that the concentrations of metabolites may not be as great in the right rumen vein as in the left rumen vein (Barncroft, McAnally and Phillipson, 1944).

In the present experiments an attempt was made to circumvent these problems by comparing the arteriovenous concentration differences to that of acetate. Quantitative estimates of absorption were then possible since the amount of acetate absorbed from the rumen daily could be estimated. This method of measuring net absorption of metabolites into the ruminal blood, then, is based on the assumption that various metabolites would be absorbed at the same rate relative to acetate in various parts of the rumen.

The accuracy of this method of estimating metabolite absorption is also dependent upon the assumption that little metabolism of acetate occurs in the rumen wall. Various workers have reported that acetate is not extensively metabolized by this tissue (Hird and Symons, 1961; Sutton et al., 1963; Hird, Jackson and Weidemann, 1966). In addition Hird and Weidemann (1964) have reported that the conversion of butyrate into ketone bodies accounted for up to 70% of the oxygen consumption of rumen tissue, indicating that butyrate is the primary substrate for metabolism in this tissue. In contrast to these results, however, Stevens (1970) and Bergman and Wolff (1971) have recently presented results which

show the possibility that up to 30% of the acetate absorbed from the rumen may not reach the portal blood in sheep; thus the estimates of absorption in these experiments may be over-estimated by this amount The accuracy of these estimates of absorption is furthermore limited by the fact that the amount of VFA produced in the rumen had to be estimated instead of measured, as was intended.

b. Estimates of rumen blood flow

The degree of accuracy of the quantitative estimates of the net absorption of various substances into the rumen venous blood can be obtained from the approximate rates of rumen blood flow presented in Table 12. Although no totally valid measurements of ruminal blood flow have been reported with which these values can be compared, Hecker and Nolan (1971) have previously estimated that blood flows past the rumen of the sheep at the rate of 800 ml/min, which is within the range of flow rates presented in Table 12. The values given in Table 12 also compare with a measurement made by Conrad et al. (1958), who found that the flow of blood into the gastrosplenic vein (the main source of which is the rumen blood) was 16.8 ml/min/kg live weight in calves.

Estimates of blood flow past the rumen can also be obtained from the rate of blood flow in the portal system of sheep. This flow rate has been measured at 43 ml/min/kg body weight (Katz and Bergman, 1969) and 32 ml/min/kg body weight (Hume, Jacobson and Mitchell, 1972). The average blood flow past the rumen in the present experiments of 33 ml/min/kg live weight thus represents a large portion of the portal blood flow, as would be expected on the basis of the anatomy of the ruminant blood system (Sisson and Grossman, 1953).

The estimated blood flows past the rumen, then, are in the range of

estimates and measurements presented in the literature. These estimates would thus be expected to give a good approximate value for the amount of material absorbed into ruminal blood daily from the rumen. In addition, the measurements of blood flow can be used to determine the factors which regulate blood flow past the rumen.

An important factor affecting blood flow in these experiments could very well have been the partial pressure of oxygen in rumen venous blood since no significant difference was observed in this value either within, or between, experiments (Table 15). The involvement of oxygen in the regulation of blood flow has been noted previously (Folkow and Neil, 1971).

A significant negative correlation ($r = -0.86$) was observed between rumen blood flow and rumen ammonia concentration, which suggests that ammonia, as well as the partial pressure of oxygen in the venous blood, influenced blood flow in these experiments. This effect may have been mediated through the partial pressure of oxygen in the venous blood, since increased ammonia concentrations would be expected to decrease the metabolic rate of the rumen tissue (Visek, 1968) and hence the rate of oxygen utilization.

B. Absorption and rumen wall metabolism of nitrogenous compounds

Ammonia was absorbed into the ruminal blood in significant amounts in these experiments. No significant correlation, however, was found between the arteriovenous ammonia concentration differences and rumen ammonia concentrations. An average of 7.1 g of ammonia nitrogen was absorbed into the ruminal blood of these sheep daily with the range of absorption being from 4.8 to 9.9 g of nitrogen daily (Table 13). Similar rates of absorption have previously been reported. McDonald (1948) concluded that from 4 to 5 g of ammonia nitrogen were absorbed

from the rumen daily. Lewis, Hill and Annison (1957) found that up to 14 g of ammonia nitrogen entered the portal blood system of sheep daily. In Section I of this thesis it was observed that 7.2 g of ammonia nitrogen appeared to be absorbed from the rumen daily in a sheep which was fed a hay diet. It is of interest to note that in the experiments of this section, an average of 7.3 g of ammonia nitrogen were absorbed into the ruminal blood of the sheep fed similar hays to that used in Section I. From these measurements and comparisons it is apparent that appearance of ammonia in ruminal blood was approximately equivalent to the rate of disappearance of ammonia from inside the rumen. It can thus be concluded that there was little formation, or utilization, of ammonia in rumen wall metabolism.

The net absorption of glycine into ruminal blood has previously been reported (Cook, Brown and Davis, 1965). It has also been noted that rumen tissue actively concentrates and transports this amino acid (Chand, Varma and Kushwa, 1968; Leibholz, 1971b). It was for these reasons, and because of the assumption that very little glycine would be formed by rumen wall tissue, that this amino acid was chosen as one of the two amino acids studied in these experiments. It was found that from 4.2 to 6.1 g of glycine nitrogen were absorbed into the ruminal blood of the sheep fed the hay 1 and straw diets, respectively, which were the only two sheep in which significant absorption of glycine was observed. Although these values for glycine absorption appear large, glycine is probably absorbed into ruminal blood faster than other amino acids (Cook, Brown and Davis, 1965).

Glutamate was absorbed from the rumen in significant amounts in both of the sheep in which glycine absorption was significant and in the

sheep fed the hay 2 diet as well. The average absorption rate of this amino acid in all experiments was 0.8 g of nitrogen daily with the range of absorption being from 0.1 to 2.2 g of nitrogen daily.

It was impossible to tell from the in vivo experiments if there was any formation of amino acids in the rumen wall, although such a possibility has been mentioned (Hoshino, Saramaru and Morimoto, 1966). Preliminary in vitro experiments were thus conducted with rumen epithelium to study amino acid synthesis by this tissue. No net formation of amino acids from ammonia was observed when rumen papilli were incubated with ammonia, 2-keto acids and energy sources. In addition, no formation of amino acid nitrogen from amide nitrogen by this tissue was detected. Glutamate and glycine were, however, formed by transamination when 2-oxoglutarate and glyoxalate, respectively, were present. Since the absorption of amino acids from the rumen has been demonstrated (Cook, Brown and Davis, 1965; Leibholz, 1971a), and since it appeared that little ammonia was metabolized in the rumen epithelium, it is thus probable that the amino acids which entered the blood in these experiments arose from the transport of amino acids across the rumen wall.

The high standard errors of estimates of the rumen arteriovenous urea concentration differences made it impossible to ascertain the degree of urea recycling in these experiments. Also no other nitrogenous compound measured in these experiments was recycled to the rumen in significant amounts, thus the source of nitrogen which entered the rumen in Section I was undetected.

C. Rumen wall metabolism

a. Metabolism of ruminal VFA

Only limited information can be gained concerning the quantitative

aspects of VFA metabolism in the rumen wall from these experiments since only estimates of production in the rumen are available. Almost all of the butyrate absorbed from the rumen, however, was metabolized to 3-hydroxybutyrate in the rumen wall in agreement with the observations of Bergman (1971). This can be concluded since little detectable butyrate was present in venous blood and since 3-hydroxybutyrate absorption into ruminal blood, when calculated relative to acetate absorption, accounted for 103, 64, 92, 200 and 100% of the estimated butyrate production in the rumen of sheep fed hay 1, hay 2, hay + ammonia, straw and barley diets respectively (Tables 11, 14).

b. Oxygen utilization by the rumen

The rate of utilization of oxygen by the rumen of the sheep ranged from 12 to 59 ml O_2/min when corrected to STP (Table 16). The rumen oxygen consumption was thus 16, 5 and 24% of the measured oxygen consumption of the sheep fed hay 2, hay + ammonia and barley diets, respectively (Table 16). No whole animal oxygen consumption measurements were made on sheep 9238, which was fed the hay 1 and straw diets, since this animal would not permit the usage of a face mask. It would be expected, however, that the percentage of the total oxygen consumption that was accounted for by rumen utilization in this animal would be higher than in the others since a larger rumen oxygen consumption was measured.

The average oxygen consumption of the rumen tissue of the three sheep was 41 ml O_2/min/kg rumen tissue (assuming the average rumen weight of the sheep was approximately 2.15% of the body weight; Ingle, Bauman and Garrigus, 1972). Since Holliday et al. (1967) have concluded that the rate of oxygen utilization by the brain and liver ranged from 35 to

48 ml O_2/min/kg tissue, it is thus possible that the rate of oxygen utilization by the rumen tissue is comparable to that of other active tissues. Another alternative to rumen tissue utilization of the oxygen taken up across the rumen, however, is that oxygen diffused from the blood into the rumen (Czerkawski and Beckenridge, 1969). Since either or both of these pathways of rumen oxygen utilization may have been operative in these sheep, no definite conclusions can be drawn from these results with respect to the mode of utilization of the oxygen consumed across the rumen.

If one-half mole of oxygen is required for the conversion of butyrate into 3-hydroxybutyrate, then the amount of oxygen utilized in the formation of 3-hydroxybutyrate was 3, 3, 9.5, 4.5 and 5.5% of the measured oxygen consumption of the rumen in the sheep fed the hay 1, hay 2, hay + ammonia, straw and barley diets, respectively. This means that only a small proportion of the oxygen removed from the blood across the rumen was used in the conversion of butyrate to 3-hydroxybutyrate.

SUMMARY

Rumen arteriovenous concentration differences of various metabolites were measured and compared to that of acetate in order to obtain a quantitative measurement of the net amount of metabolites absorbed into the rumen blood daily or utilized by the rumen daily.

From 4.8 to 9.9 g of ammonia nitrogen were estimated to enter the blood daily from the rumen. The amino acids glycine and glutamate were also absorbed into the rumen blood in significant amounts in three of the five experiments. It was concluded that these two amino acids, which were absorbed into ruminal blood at the rate of from 0.2 to 7.1 g nitrogen daily when absorption was significant, probably arose from inside the rumen rather than from formation in the rumen wall since little ammonia appeared to be fixed in the transfer of ammonia across the wall and no net synthesis of amino acids by the rumen wall was detected *in vitro*.

The oxygen consumption of the rumen was estimated at 12 to 59 ml O_2/min (STP). This oxygen could have been utilized by rumen tissue or could have diffused into the rumen from the blood stream.

GENERAL DISCUSSION

The digestion and assimilation of foods in ruminant animals is, to a large extent, dependent upon rumen metabolism. The experiments in this thesis were thus designed to study the metabolism of nitrogenous compounds in the rumen and to examine the interrelationships between rumen and animal tissue metabolism.

In the present experiments it was found that the apparent digestibility of dry matter, nitrogen and combustible energy of plant material in the rumen and in the whole animal were similar. These results, although they show extensive metabolism of nitrogenous and other compounds in the rumen were obtained under steady state conditions. It is probable that different feeding conditions could result in more plant material escaping rumen degradation. It has, in fact, been observed that considerable protein can pass through the rumen without being fermented (McDonald, 1954; Hogan and Weston, 1969b).

It was observed that the entry rate of nitrogen into the rumen ammonia pool was equivalent to 60 to 92% of the nitrogen intake (Section I). Similar results were obtained by Pilgrim, Gray and Belling (1969) and Nolan and Leng (1972). Thus, a large portion of the nitrogenous compounds which are degraded in the rumen yield ammonia as an end product of metabolism.

Rumen ammonia was also found to be a major source of nitrogen for rumen bacteria since it was observed that from 50 to 78% of the bacterial nitrogen was derived from ammonia in the rumen of sheep (Section I; Pilgrim *et al.*, 1970). Protozoa derived from 31 to 64% of their nitrogen from rumen ammonia (Section I; Pilgrim *et al.*, 1970). It appeared that, as rumen ammonia concentrations increased, less of the microbial

nitrogen originated from ammonia.

Since little food nitrogen that could be available to the host remains undigested in the rumen, and nitrogen which is not utilized by rumen microorganisms is largely converted to ammonia, the extent of microbial growth in the rumen is of fundamental importance to the protein nutrition of the ruminant animal (Milligan, 1967). The experiments described in this thesis showed that from 8.6 to 13.2 g of protein were incorporated into microbial material for every 100 g of dry matter digested in the rumen under steady state conditions (assuming that microbial nitrogen is 81% protein nitrogen, Walker and Nader, 1970). Similar estimates of microbial growth have been obtained by Pilgrim et al., (1970). The efficiency of microbial growth has been found to be affected by the diet of the animal (Hume, Moir and Somers, 1970), thus it could very well be that the yield of microbial protein in the rumen would limit the growth of ruminant animals under at least some circumstances.

The efficiency of microbial growth observed in Section I and that reported by Hume (1970) were greater than would be expected on the basis of anaerobic in vitro experiments (Hungate, 1966). This suggested that microbial growth in the rumen might occur under partly aerobic conditions, since there is the possibility that oxygen could diffuse into the rumen from the blood stream (Czerkawski and Beckenridge, 1969). This possibility was examined in Section IV of this thesis. Oxygen utilization by the rumen was measured at the rate of from 12 to 59 ml O_2/min at STP. This accounted for up to 24% of the measured total oxygen consumption in one sheep. It could not be determined, however, whether this oxygen diffused into the rumen or was utilized in rumen tissue metabolism.

As well as obtaining nitrogenous compounds from the rumen via the intestinal tract, the ruminant animal also absorbs nitrogenous compounds from the rumen into the ruminal blood stream. In agreement with reports of other workers (Cook, Brown and Davis, 1965; Leibholz, 1971a), it was suggested that amino acids were absorbed from the rumen in the present experiments. In addition, no significant formation of amino acids by rumen tissue was observed *in vitro*. Up to 7 g of ammonia nitrogen were lost from the rumen by absorption (Section I) and this corresponded to the estimates of the amount of ammonia appearing in the ruminal blood stream (Section IV). This suggests that little net transformation of ammonia occurred in the rumen wall.

Absorbed rumen ammonia is converted to urea by the liver (McDonald, 1948). The extent of this conversion was found to be approximately 90% of the ammonia in portal blood when a diet relatively high in protein was fed (Section III). It is probable that less ammonia would be converted into urea when diets with a lower protein content are consumed (Sprinson and Rittenberg, 1949).

In an effort to detect the non-urea sources of nitrogen entering the rumen when low-protein diets are fed (Section I; Harris and Phillipson, 1962; Weston and Hogan, 1968) the possibility of nitrogen gas fixation in the rumen was studied using $^{15}N_2$. From *in vitro* incubation results, it was concluded that any nitrogen gas fixation would be of very limited importance to the ruminant animal. Further investigations into the routes of entry of nitrogenous compounds into the rumen were limited because of the analytical problems involved. It was found, however, that neither glycine nor glutamate were lost from the ruminal blood into the rumen. The non-urea source of nitrogen which enters the

rumen when low nitrogen diets are fed thus remains to be identified.

Another characteristic of ruminant nitrogen metabolism which was unresolved in the present experiments concerns the rapid appearance of ^{15}N in amino acid, amide and protein nitrogen in ruminants that has been observed when ^{15}N-ammonia was present in the rumen (Land and Virtanen, 1959; Faust et al., 1966; Abe and Kandatsu, 1969). Liver and rumen wall metabolism were studied in an effort to explain this aspect of nitrogen metabolism, however, it was found that 93% of ammonia reaching the liver was converted to urea (Section III) and that there was little net formation of non-ammonia compounds from ammonia in the rumen wall (Section IV).

The observed incorporation of ammonia nitrogen into other nitrogenous compounds could be explained if a dynamic exchange existed between nitrogenous compounds, particularly in the rumen or rumen wall. Schoenheimer, Ratner and Rittenberg (1939) have concluded that exchange reactions do occur in the intestinal wall of monogastric animals. Such a dynamic exchange is indicated for ruminant animals by the observations that bacterial nitrogen in the rumen and plasma urea nitrogen have similar enrichments when ^{15}N-ammonia is infused into the rumen (Section I; Nolan and Leng, 1972), and that ammonia and urea nitrogen have similar ^{15}N enrichments for the period from 8 to 24 hours after administration of a single injection of ^{15}N-ammonia to the rumen of sheep (Nolan and Leng, 1972). Further evidence supporting the concept of a dynamic exchange between the nitrogen of various compounds can be derived from results of Nolan and Leng (1972), who found that considerable recycling occured in the rumen nitrogen pool. In addition, the seemingly slow turnover time in relation to other reported values of bacterial

protein nitrogen as estimated by the rate of incorporation of ^{15}N-ammonia into microbial nitrogen (Section I) suggests that the bacterial nitrogen was in equilibrium with a pool of material which turned over more slowly than the microbial nitrogen.

The results presented in this thesis show that several aspects of ruminant metabolism should be examined in more detail. Among these are factors affecting the efficiency of microbial growth in the rumen, amino acid absorption from the rumen, rumen tissue metabolism, the nitrogen sources of rumen protozoa and factors affecting ruminal blood flow and oxygen consumption. Knowledge concerning these aspects of metabolism would be beneficial in understanding and meeting the nutritional needs of ruminant animals.

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APPENDIX A

Establishment of an Abomasal Fistula in Sheep

Reference: Ash, 1962.

Two sheep were fitted with abomasal fistulae in these experiments. In preparation for the surgical operation feed and water were withheld from the sheep for a period of 24 hours. The animals were then clipped and shaved to the skin surface in those areas where incisions were to be made. They were anaethetized with Nembutal administered through a jugular catheter. A tube was inserted into the trachea of the sheep on the operating table to prevent fluid from entering the lungs.

An incision, approximately 25 cm in length, was made slightly to the right side of the midline, using aseptic techniques. The abomasum was located and pulled through this incision. Two circular purse-string sutures, one slightly inside the other, were made in the wall of the abomasum. The wall of the organ was then cut and a plexi-glass cannula was inserted. The purse-string sutures were then tightened around the flange of the cannula and the barrel of the cannula was exteriorized between the 11th and 12th rib on the right side by removing a 5 cm section of the 10th rib.

Soft cotton pads were placed next to the animal's body and a plexi-glass washer was turned down over the threads on the barrel of the cannula to keep it in place. A cap was routinely kept over the barrel of this cannula.

After surgery, the animals were treated with 4cc of penicillin daily for 4 days to control infection.

One sheep, fitted with a cannula in this manner, has remained

healthy for a period of 3 years. Another ewe with an abomasal fistula had to be destroyed after 18 months because of spreading of the ribs around the cannula and resulting infection.

APPENDIX B

Separation of Bacteria and Protozoa

Reference: Blackburn and Hobson, 1960b.

1. Approximately 100 ml of ruminal or abomasal fluid was strained through six to eight layers of cheesecloth. The residue from the cheesecloth was resuspended in 100 ml of 0.85% (w/v) NaCl saturated with chloroform, and restrained through cheesecloth. The two solutions were combined, placed on ice and transported to the lab.

2. The diluted ruminal or abomasal fluid was filtered through cotton cloth (27 threads per cm), then centrifuged at 114 x g for 5 minutes. The deposit, or protozoal fraction, was resuspended, washed and centrifuged in 0.85% (w/v) NaCl.

3. The supernatant fraction from the first centrifugation (above) was spun at 19000 x g for 20 minutes, the supernatant was discarded and the deposit was washed by centrifuging in saline. This deposit was considered to be the bacterial fraction.

APPENDIX C

Isolation of Microbial Protein

Reference: Roberts et al., 1955.

1. The separated protozoal or bacterial cells from approximately 10 ml of rumen fluid were mixed with 10 ml of cold, 5% (w/v) trichloroacetic acid. After 30 minutes at 5 C the suspension was centrifuged at 2000 x g and the supernatant was discarded. Residual fluid was removed from the walls of the centrifuge tubes with a cotton swab.
2. The precipitate obtained above was mixed with 10 ml of 75% (w/v) ethanol and heated to 40 - 50 C for 30 minutes. This preparation was centrifuged at 2000 x g and the supernatant, which contained some soluble protein, was saved and extracted twice with 10 ml of diethyl ether (water was added to the tube in these two extractions to cause the separation of the two layers). The ethanol-water fraction was then removed and the soluble protein was dried and saved for ^{15}N analysis.
3. The precipitate from the extraction procedure above (step 2) was suspended in a mixture of 5 ml of diethyl ether and 5 ml of 75% (w/v) ethanol and kept at 40 - 50 C for 15 minutes then centrifuged.
4. Ten ml of 5% (w/v) trichloroacetic acid was added to the precipitate obtained above and the mixture was heated in a boiling water bath for 30 minutes and then recentrifuged.

5. The remaining precipitate was washed in acidified 75% (w/v) ethanol, centrifuged, resuspended in diethyl ether and centrifuged again.

6. The protein precipitate was combined with the protein isolated in step 2 and then analysed for ^{15}N.

APPENDIX D

^{15}N Sample Preparation

Reference: Mulligan and Workmall, 1959.

1. Two and one-half g K_2SO_4, 2.5 ml $HgSO_4$ (made by dissolving 10 g of red HgO in a solution of 12 ml of concentrated H_2SO_4 added to 100 ml of H_2O) and 5 ml of concentrated H_2SO_4 were added to a sample containing approximately 20 mg of nitrogen in a 100 ml micro Kjeldahl flask.

2. The mixture was boiled under close supervision until the solution became clear and was then left for a total digestion time of 16 hours.

3. Following digestion, the sample was cooled and 10 ml of H_2O, 25 ml of 10-N NaOH and 1 g of zinc dust were added.

4. The ammonia was distilled through water-cooled condensers into 0.1-N H_2SO_4 along with a volume of 30 ml of H_2O.

5. The ammonium sulfate solution obtained from the distillation procedure was reduced in volume to approximately 1/2 ml by boiling on a hotplate.

6. This concentrated solution was placed in a two compartment, Y-shaped vessel. Sodium hypobromite (0.1% KI added) was placed in the other compartment.

7. The solutions were frozen in liquid nitrogen and the pressure in the vessel was reduced to less than 10^{-3} mm of Hg with a mercury diffusion pump.

8. The stopcock was closed and the vessel was removed from the vacuum system. The two solutions were mixed and nitrogen gas was liberated.

9. The nitrogen gas was sealed in a break-seal tube and stored until it could be analysed for ^{15}N on the mass spectrometer.

APPENDIX E

Catheterization of the Ruminal Vein

Eight sheep had catheters placed in the right ruminal vein for these experiments. The catheters remained patent for periods varying in length from a few hours to 2 months when flushed twice a day with heparin in isotonic saline (1000 IU/ml).

Surgery was performed after the animals had been without food and water for a period of 24 hours. All surgery was carried out under aseptic conditions with intravenously infused Nembutal used as the anaesthetic. The anaethesized animal was placed on its right side on an operating table and a tube was inserted into the trachea to prevent any fluid from entering the lungs. An incision 25-30 cm long and approximately 2.5 cm posterior, and parallel to the last rib was made on the left side of the animal. The rumen of the animal was pulled out through this incision so that the large right ruminal vein could be found in the right longitudinal groove of the rumen.

This vein was catheterized, using Dow Corning Medical Grade Silastic Tubing 0.076 cm I.D. and 0.165 cm O.D., by puncturing the vein with a 13 gauge needle and inserting the catheter into the vessel through the needle. The catheter was inserted a distance of 15 cm into the vein and was secured to the rumen wall with nylon sutures. One suture was placed loosely around the catheterized vein and the rest of the tubing was then fastened to the rumen wall in the form of a semi-circle with a diameter of approximately 2.5 cm with six to eight stitches used in total. It was found that this method of securing the catheter to the rumen wall prevented the catheter from being removed from the ruminal vein.

The rumen was returned to the body cavity and the catheter was exteriorized through an opening made with a 13 gauge needle dorsal to the incision. Approximately 30 cm of catheter was pulled through the skin and approximately 75 cm of the catheter was left inside the animal body. The incision was then closed by suturing like layers of tissue together. Daily, intramuscular administration of 4 cc of penicillin was maintained for a period of 4 days after the operation was performed to control infection.

Sheep which were killed and examined weeks after surgery showed no inflammation around such a catheter and there was no detection of any adhesions between the rumen and the abdominal wall.

APPENDIX F

Chemical Determinations

a. The determination of polyethylene glycol

Reference: Smith, 1959b.

1. Rumen fluid was strained through two layers of cheesecloth and centrifuged at 2000 x g for 5 minutes.

2. One ml of the rumen sample, or 2 ml of abomasal sample, was transferred to a clean, Neoprene 40 ml test tube and made up to 11 ml with deionized H_2O.

3. In the order given, 2 ml of 10% (w/v) $BaCl_2.2H_2O$, 2 ml of 4.725% (w/v) $Ba(OH)_2.8H_2O$ and 2 ml of 5% (w/v) $ZnSO_4.7H_2O$ were added. After each addition the preparation was mixed with a Vortex test tube mixer.

4. Following the final mixing the material was allowed to stand for 5 minutes, then the tubes were centrifuged at 2000 x g for 15 minutes.

5. Three ml of the supernatant from each tube were then pipetted into a Bausch and Lomb colorimeter tube and 3 ml of 30% (w/v) trichloroacetic acid containing 5.9% (w/v) $BaCl_2.2H_2O$ were added. The contents were quickly and thoroughly mixed by inversion.

6. The tubes were left for exactly 20 minutes and were read at 525 nm with a Bausch and Lomb Spectronic 20.

b. The determination of lignin

References: Norman and Jenkins, 1934.
Thomas and Armstrong, 1949.

1. Duplicate samples of approximately 1 g of finely ground feed, dried feces or dried abomasal material was weighed on Whatman filter paper (number 42). This paper was folded and placed in extraction thimbles (Whatman single thickness, 25 x 80 mm).

2. The sample was extracted for 2 hours with a mixture of ethanol and benzene (1:1, v/v) in a Soxhlet apparatus.

3. The extracted material was quantitatively transferred to 600 ml beakers, 100 ml of 5% H_2SO_4 (w/w) was added and the solution was refluxed for 1 hour on a Goldfisch apparatus.

4. The hot material was quantitatively filtered through hardened filter paper (Whatman number 52) by rinsing with hot H_2O.

5. The residual material on the filter paper was quantitatively transferred to a weighing bottle (2.5 X 4.75 cm) with a fitted ground glass cap and 10 ml of 72% (w/w) H_2SO_4 was added. The material was allowed to digest for 2 hours at 20 C.

6. The solution was quantitatively transferred to a 600 ml beaker with 230 ml of H_2O to give a final solution of 3% (w/v) H_2SO_4.

7. This diluted sample was refluxed for 3 hours and was then filtered through pre-ashed, pre-weighed Gooch Crucibles and washed with hot H_2O.

8. The crude lignin samples were dried overnight at 105 C under reduced pressure, cooled, weighed, then ashed at 600 C overnight.

9. Lignin in the sample was corrected to an ash-free basis and reported as a percentage of the total dry matter in the sample.

c. The determination of ammonia and urea

Reference: Fawcett and Scott, 1960.

1. Duplicate samples were placed in test tubes which had been rinsed with deionized H_2O and the volume was made up to 1 ml with deionized H_2O.

2. One ml of urease solution (30 mg of special purity urease substantially ammonia free, were added to 1 litre of buffer made by dissolving 0.06 g of KH_2PO_4 and 0.02 g of Na_2HPO_4 in 1 litre of solution) was added to the tubes and the tubes were mixed and allowed to stand for 30 minutes.

3. The following solutions (made with deionized water) were added to the test tubes immediately after each other in the order given: 2 ml of sodium phenate (25 g of phenol and 12.480 g of NaOH diluted to 1 litre), 3 ml of 0.01% sodium nitroprusside and 3 ml of 0.02 N sodium hypochlorite.

4. The tubes were mixed and the color was allowed to develop for at least 1/2 hour in darkness.

5. The optical density was read at 630 nm on a Bausch and Lomb Spectronic 20. Those readings were corrected for blank readings and compared to a standard curve to obtain the concentration of ammonia from urea in each tube.

In the determination of ammonia step 2 (incubation with urease) was ommitted.

d. The determination of glycine

References: Alexander, Landwehr and Seligman, 1945.
Christensen, Riggs and Ray, 1951.

1. A sample of deproteinized blood or standard was placed in a 25 ml round bottom flask and made up to 4 ml with deionized H_2O.

2. Two ml of phosphate buffer (200 g KH_2PO_4 and 35 g K_3PO_4 per litre: PH 5.5) and 1 ml of a 2% (w/v) ninhydrin solution were then added.

3. The flask was connected to an all glass condenser and approximately 6 ml of aqueous material was distilled into a graduated test tube using a free flame from a bunsen burner. The total distillation time for this procedure was less than 3 minutes.

4. After the flame was removed and the flask had cooled slightly, it was cooled to room temperature by immersing it in cool water.

5. Three ml of H_2O was then added and the distillation was continued to dryness.

6. The distillate collected in the receiving tube was made up to 10 ml with H_2O.

7. Five ml of this solution was taken and placed in a test tube cooled in ice. Four ml of concentrated H_2SO_4 was added, the material was mixed, then cooled to room temperature and 0.1 ml of 5% (w/v) chromotropic acid was added.

8. The tubes, after mixing, were covered and placed in a boiling water bath for 30 minutes.

9. The optical density of the solution was determined within 1 hour after the tubes were cooled to room temperature by using a Bausch and Lomb Spectronic 20 and reading at 575 nm (extinction coefficient is 1.58×10^4).

e. The determination of glutamate

Reference: Bernt and Bergmeyer, 1965.

1. A solution of 0.2 ml of a glycine-hydrazine buffer, pH. 9.0 (0.75 M glycine and 0.6 M hydrazine) was placed in a cuvette and 100 µl of sample and 20 µl of NAD (3×10^{-2} M: Sigma Chemical Co., Grade III) were added.

2. When a constant optical density reading was obtained at 340 nm (Gilford Recording Spectrophotometer: Model 2000) 10 µl of a glutamic dehydrogenase solution (Sigma Chemical Co.: Type II in 50% glycerol and substantially free from ammonium ions) was added.

3. The optical density of the solution was measured every minute at 340 nm until the reaction was complete.

4. The change in absorbance, corrected for the blank, was used to calculate the amount of (1)-glutamate in the sample cuvette according to the following formula:

$$\mu \text{ moles (1)-glutamate} = \frac{\text{change in optical density} \times 0.330}{6.22}$$

f. The determination of 3-hydroxybutyrate

Reference: Williamson and Mellanby, 1965.

1. An amount of 100 µl of 0.1 m Tris buffer (pH 8.5), 100 µl of 2.5% (v/v) hydrazine hydrate (pH 8.5), 100 µl of sample and 20 µl of NAD

(1.3×10^{-2} M: Sigma Chemical Co., Grade III) were added to a cuvette and mixed.

2. When a constant optical density reading was obtained at 340 nm (Gilford Recording Spectrophotometer; Model 2000), 3 ul of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30; 2/mg/ml; Boehringer-Mannheim Co.; Grade 1) was added and the solution was mixed.

3. The optical density of the solution was measured every minute at 340 nm until the reaction was complete.

4. The change in absorbance, corrected for the blank, was used to calculate the amount of 3-hydroxybutyrate in the sample cuvette according to the following formula:

$$\mu \text{ moles D-(-)-3-hydroxybutyrate} = \frac{\text{Change in optical density} \times 0.323}{6.22}$$

g. The determination of hemoglobin

Reference: Hycell cy nmethemoglobin determination. Hycell Inc. P.O. Box 36329 Houston, Texas 77036.

1. A 20 µl sample of whole blood was added to 5 ml of Hycell cyan-methemoglobin reagent and the tube was mixed.

2. The absorbance of the solution was determined with a Bausch and Lomb Spectronic 20 set at 540 nm.

3. The concentration of hemoglobin was determined from a standard curve prepared from a Hycell Cyanmethemoglobin standard.

APPENDIX G

Setting used for the Mark I Nuclear Chicago Liquid Scintillation System

	Channel A	Channel C
Attenuator	C_{550}	D_{550}
Window	L-U	L-U
Lower Discriminator	0.5	0.5
Upper Discriminator	2.9	9.9